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AN INVESTIGATION OF THE VALIDITY OF DEATH CERTIFICATION OF CANCER OF THE LUNG IN LEEDS

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AN enumeration of new cases of lung cancer (*i.e.* cancer of the trachea, pleura, lungs or bronchi) diagnosed in 3 hospital centres in Great Britain during the years 1948–1952 was made previously (Bonser and Thomas, 1955). It was noted that more deaths were recorded than cases were diagnosed in the hospitals in two regions—Aberdeen and Leeds City. However, the figures were not strictly comparable, as not all the cases identified clinically in any year would die in the same year, though the overlap would tend to balance over the five-year period. In the Aberdeen region, approximately one-fifth of recorded deaths from lung cancer did not appear in the hospital records ; in Leeds City one-tenth. But in the latter area, there was a greater discrepancy between recorded female deaths and female cases diagnosed in hospital, approximately one-third of recorded female deaths not appearing in the hospital records. The reason for this was obscure unless it happened that more deaths from secondary tumours of the lung were included in the female deaths.

The present investigation was undertaken in order to obtain more accurate information about the relation between the numbers of recorded deaths from lung cancer and the numbers of cases diagnosed clinically. Leeds is a city of approximately half a million inhabitants, with two large general hospitals. In one of these, the Leeds General Infirmary, which is the teaching centre, there is a thoracic unit to which many cases of suspected malignant thoracic disease are referred either by general practitioners or from other hospitals ; in the other, St. James's Hospital, a regional board hospital with its own out-patient department, there is no thoracic unit but both acute and chronic beds are available. No thoracic surgery is undertaken at this hospital. There are other smaller hospitals in Leeds and a Chest Clinic, to which cases are referred by general practitioners for diagnosis and from which they can be referred to the two large hospitals for special investigation and treatment. Originally mainly concerned with tuberculosis, this clinic deals with all varieties of chest disease.

The present survey comprises two parts :—

(1) A scrutiny of death certificates for the years 1950–54, coded as 162 and 163, *i.e.* cancer of trachea, pleura, lungs or bronchi. This information was obtained from the Statistics department of the Medical Officer of Health for Leeds. Certificates for the years 1955 to 1958 were also scrutinised, but not included in the

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survey, in order to cover the clinical cases diagnosed during the 1950–54 period but surviving beyond it.

(2) All the cases recorded in the two main Leeds Hospitals, the General Infirmary and St. James's Hospital, indexed in the disease index under 162 and 163 (International Statistical Classification, 1948) were scrutinised and classified according to the firmness of the diagnosis. Those for the years 1948–52 had been collected in the former survey ; those for 1953 and 1954 were now added.

Scrutiny of death certificates

Information about death certificates is filed in two places : (1) the office of the Medical Officer of Health for each County Borough and (2) the General Register Office in London. When inquiry was made, it was found that 1,029 cases of lung cancer (code no. 162 and 163) had been filed in the Statistics Department of the Medical Officer of Health for Leeds during the years 1950–54 inclusive. After special search, an additional 7 certificates were found, but there was still a discrepancy of 15 cases between this total of 1,036 and that of the General Register Office, where 1,051 deaths from this cause were recorded in this period. The suggested reasons are as follows : in the General Register Office, all death certificates are coded without reference to the coding of the Medical Officer of Health's staff ; inquiries are also made about ambiguous cases, and a few clerical errors are certain to occur in both groups. We decided, for practical reasons, to use the certificates filed by the Statistics department of the Medical Officer of Health for Leeds. Inquiry to the Registrar General failed to find a means of tracing the extra 15 cases coded by his staff, because it is the practice to discard the coding records when 4 years have elapsed. When re-coding of non-tallying clinical cases (p. 9) was undertaken in the General Register Office, it seemed probable that 7 of these cases could be accounted for by differences in coding between the staffs of the Medical Officer of Health for Leeds and of the Registrar General.

Estimate of extent of agreement with clinical diagnosis

The death certificates were paired with the clinical case records obtained from the hospitals, but a large surplus of death certificates remained. Using the names and addresses on the certificates, a search was then made through the Admission Index of each hospital and a further group of pairs with clinical cases thus emerged. The reasons for their original non-appearance in the hospital disease index (code no. 162 and 163) were :—

(*a*) cases diagnosed in the out-patient department are not included in the disease index.

(*b*) on occasion, the provisional diagnosis on admission had not been amended to the final diagnosis of carcinoma of the bronchus.

(*c*) there was a misunderstanding of the diagnostic wording used by the medical staff. For example, " pathological fracture of the femur " did not appear under code no. 162 or 163, although autopsy had revealed that the primary tumour was in the bronchus.

(*d*) clerical errors and omissions, *i.e.* cases which did not emerge from the punch-card throw-out or were missed out of the list supplied.

Some death certificates still remained and these were traced back through the other hospitals and clinics in Leeds, or occasionally outside the County Borough.

Coroner's reports were scrutinised and finally a residue (21 cases) was traced by inquiry to the certifying general practitioner responsible for the diagnosis and certification. Some of the 37 untraced cases were probably certified in this way but we have no means of knowing how many.

A decision had to be made as to whether those cases not diagnosed in life by clinical methods but revealed at autopsy should be included in the "clinically diagnosed" cases. It was decided that, for the purpose of this survey, they should be so included, as it was primarily desired to test the accuracy of death certification. Death certificates based on autopsy findings are the most likely of all to be accurate.

Of 1,036 death certificates, there were only 37 in which it was impossible, by the use of all the methods described above, to discover how or where diagnosed. The agreement between death certificate and clinical diagnosis was very high (96·5 per cent, Table I). Errors and untraced cases were proportionately higher in females. One-third of such cases were females, whereas in the tallied cases the female incidence was only one-sixth.

TABLE I.—*Comparison of Death Certificate With Clinical Diagnosis*

	Male	Female	Male	Female	Total No.	Total Per cent	Ratio M. : F.
Tally with clinical diagnosis	836	128	—	—	964	96·5	7 : 1
Including :							
Confirmatory autopsy	—	—	151	18	—	—	—
Not diagnosed in life but revealed at autopsy	—	—	84	14	—	—	—
Notes lost but diagnosis known	—	—	10	1	—	—	—
Non-tally with clinical diagnosis	25	10	—	—	35	3·5	3 : 1
Total certificates	861	138	—	—	999	—	—
Untraced by any means	28	9	—	—	37	—	3 : 1

The reasons for the 35 errors in certification were as follows :—

(*a*) Failure to make use of the final post-mortem diagnosis for the purpose of death certification (8 males, 1 female). Three of these had been diagnosed clinically and 6 had not. In all of them the primary tumour was thought on naked-eye inspection to be in the bronchus but on histological examination it was proved to be in another site. The other site was the prostate in 7 cases.

(*b*) Failure to make use of the immediate post-mortem diagnosis for the purpose of death certification (8 males, 3 females). In 5 of these there was a tentative clinical diagnosis of carcinoma of the bronchus, but in the remaining 6 no clinical diagnosis had been attempted. All were certified as dying of carcinoma of the bronchus but another cause was proved at post-mortem. Probably the certificate was issued before the autopsy was done.

(*c*) Three cases died at home (2 males, 1 female) and were certified by the practitioner as dying of carcinoma of the bronchus although a firm diagnosis of another clinical condition had been made in hospital.

(*d*) In 12 cases (7 males, 5 females) the cause of death was certified as cancer of the lung, but it was clear that the lung tumour was metastatic from a primary elsewhere, as was proved by autopsy in 7 cases. The death certificate might have

been issued before the post mortem was done or the wording " cancer of the lung " might have been used in the sense of metastatic cancer.

TABLE II.—*Actual Cause of Death in 35 Cases Incorrectly Certified at Death*

Sex	Non-cancer	Cancer: Site of primary tumour	Without lung metastasis	With lung metastasis
Male	6 (6)	Carcinomatosis	—	1 (1)
		Prostate	—	10 (9)
		Other	3 (2)	5 (3)
Female	3 (3)	Breast	—	3 (1)
		Ovary	—	1
		Other	—	3 (2)

Figures in brackets indicate numbers on which autopsy was performed.

In Table II, an analysis is made of the true causes of death among the 35 cases erroneously certified as dying from cancer of the bronchus. In approximately one-quarter, no cancer was present ; in the remaining three-quarters metastatic lung cancer was present in the majority of cases of both sexes, the prostate usually being the primary site in the males and the breast or ovary in the females. In these very small numbers there was no sex difference in the proportion of incorrectly diagnosed cases where the true cause of death was metastatic cancer.

Estimate of the accuracy of the data on which the certificates were based

By international agreement (Symposium on the Endemiology of Cancer of the Lung, 1953) the validity of the clinical diagnosiss of carcinoma of the lung was assessed in three classes (Table III). The hospital notes of the cases in the present

TABLE III.—*The Validity of the Diagnosis of Carcinoma of the Lung Decided by International Agreement*

Class	Nature of the examination
I	Histology of primary tumour or Histology of secondary tumour, where primary tumour in lung is certain and diagnosed by : (*a*) radiological examination (*b*) bronchoscopy (*c*) thoracotomy or Cytology (sputum or pleural fluid), where primary is certain and diagnosed by (*a*), (*b*), or (*c*) or Autopsy
II*	Cytology only or Radiology only or Bronchoscopy only or Thoracotomy only } without biopsy
III	Case history and physical examination (without further study)

* In the Leeds cases these ancillary methods of diagnosis were usually combined.

survey, however, were individually scrutinised by an experienced doctor and it was found, especially amongst the patients attending the thoracic unit of the teaching hospital, that it was frequently the practice of the consultant-in-charge to sum up

the whole case and to make a firm diagnosis even in the absence of histological examination or autopsy. We feel that weight must be attached to such opinion.

Four hundred and seventy-seven of 954 cases belonged to class I (Table IV) which represents 50 per cent of the total. Of these, well over a half received a post mortem examination. In 169 cases, this confirmed the clinical diagnosis already made, but in 98 cases the autopsy revealed the true diagnosis. In these 267 autopsy records, histological confirmation was made in two-thirds.

TABLE IV.—*Estimate of the Degree of Accuracy of Death Certification According to the Validity of the Clinical Diagnosis*

Category	Means of diagnosis	Male	Female	Total No.	Total Per cent	Ratio M. : F.
I	Clinical histology	181	29	210		
	Confirmed by autopsy	151	18	169		
	Diagnosed by autopsy	84	14	98		
	Total	416	61	477	50·0	6·8 : 1
II	Ancillary methods without histology	372	57	429*	45·0	6·5 : 1
III	Physical examination and history only	38	9	47	5·0	4·2 : 1
	Total	826	127	953		

* Includes 333 cases in which the diagnosis was firm even without histological confirmation.

In another 333 cases (included in class II, Table IV), the surgeon or physician was confident that a diagnosis of carcinoma of the lung was correct, taking into account all the findings, which brings the percentage of highly valid cases to 85 per cent. Ancillary evidence, without histology, formed the basis of diagnosis in 429 cases (45 per cent) and physical examination of 47 (5 per cent). In the latter, which was the least adequately diagnosed group (Table IV), there was a greater proportion of females, but the differences are not statistically significant. The data do, however, fit with thosegiven in Table I, where it was noted (p. 3) that females formed a high proportion of errors and untraced cases.

Analysis of the clinical diagnosis in cases certified as lung cancer at death and submitted to post mortem examination

An analysis was undertaken of the kind of clinical diagnosis which was made in the 169 confirmatory autopsies (Table I), the 98 autopsies which revealed the condition for the first time and the 27 autopsies where death certification had been incorrect (Table V). If the 13 cases of sudden death are omitted, the autopsy being ordered by the Coroner and the lung cancer being the actual cause of death, 281 cases remain. In 169 cases (60 per cent) the clinical diagnosis was confirmed, and in 123 of these (76 per cent) the clinical diagnosis had been made by ancillary methods (class II). In 85 cases (30 per cent) lung cancer was revealed for the first time. But in 37 (nearly half) of these, no attempt at diagnosis was made, because the patient was usually too ill and in hospital for too short a time for a complete examination to be made. There were 27 cases (10 per cent) of positive

TABLE V.—*Clinical Diagnosis in 294 Cases Certified as Dying of Cancer of the Lung and Submitted to Autopsy*

Type of autopsy	Clinical class	Male	Female	Total
Confirmatory	I	30	9	39
	II	115	8	123
	III	6	1	7
	Total	151	18	169
Lung cancer revealed	Another condition diagnosed—			
	Malignant	15	4	19
	Non-malignant	24	5	29
	No attempt at clinical diagnosis	33	4	37
	Sudden death (autopsy ordered by Coroner)	12	1	13
	Total	84	14	98
Lung cancer *not* revealed	I	0	1	1
	II	6	2	8
	III	1	0	1
	No written evidence for clinical diagnosis	14	3	17
	Total	21	6	27

clinical mis-diagnosis. Only one of these belonged to class I, and 9 to classes II and III. The remaining 17 cases were accounted for by the fact that the death certificate was written before the post mortem was done.

Willis (1948) discussed the errors of cancer diagnosis as revealed by necropsy at the Alfred Hospital, Melbourne, and divided the cases into 5 classes :—

A. Clinical diagnosis of cancer of the lung, verified by necropsy.

B. No malignant disease diagnosed clinically; cancer of the lung revealed by necropsy.

C. Malignant disease diagnosed clinically, but primary site incorrect or unspecified; cancer of the lung diagnosed by necropsy.

D. Erroneous clinical diagnosis of cancer of the lung; some other primary tumour being disclosed by necropsy.

E. Erroneous clinical diagnosis of cancer of the lung; no malignant tumour of any kind found at necropsy.

In the present survey, 338 autopsies were performed on cases of cancer of the bronchus in which the diagnosis was either written on the clinical notes or on the death certificates. An analysis of the validity of diagnosis according to Willis's scheme is made in Table VI. Account must be taken of the selected nature of the material. The more complex cases are the ones most likely to come to autopsy,

TABLE VI.—*Analysis of Clinical Diagnosis in 338 Autopsy Cases of Carcinoma of the Lung*

Correct clinical diagnosis A			Incorrect clinical diagnosis								Ratio $\frac{D+E}{A}$
			B		C		D		E		
M.	F.	Total per cent	M.	F.	M.	F.	M.	F.	M.	F.	(approx.)
162	22	54	84	14	23	6	14	3	7	3	1 : 7

while the straightforward cases are discharged from hospital or omitted from autopsy. No assessment can be made of how many extra cases would be revealed if autopsy was done on all deaths and this is the information which it would be most valuable to secure.

The actual number of cases of cancer of the lung is the sum of $A + B + C$, which is 311 or 92 per cent. One hundred and eighty-four (54 per cent of the total) were diagnosed correctly clinically, the remaining 127 being revealed at autopsy. Naturally, in many of these cases the disease had been suspected in life but autopsy confirmation was awaited before the clinical diagnosis was finally decided. In only 8 per cent of the total cases was a false positive diagnosis made ($D + E$). Willis points out that these errors of inclusion are more serious statistically than errors of omission ($B + C$) because they falsify the data in a positive way. If they attain a large proportion in any series of cancer cases, analysis of properties such as age, sex, social and racial incidence, clinical course or response to therapy would be placed on a false basis. The ratio $D + E : A$, being low in this series, shows that for every 7 correct diagnoses, there was only one false positive diagnosis.

There are cases in which the tentative diagnosis of bronchial cancer may have been recorded in the notes but the autopsy diagnosis revealing some other cause of death would supplant the original clinical diagnosis. There is no means of tracing such cases in the hospital records as they are indexed according to the autopsy findings, but had it been possible to trace them, they would have been included in D and E. This would have raised the $\frac{D + E}{A}$ ratio.

Scrutiny of the clinical cases

The aim was to find out how many of the cases living in Leeds city, and diagnosed by all the available means in two hospitals during the years 1950–54, were certified at death and then indexed by the Medical Officer of Health as having died of cancer of the lung.

The method of search was to consult the disease index (code no. 162 and 163) of the two main hospitals and to pair the names thus obtained with their death certificates. As the latter were available for the years 1955–57, it was expected that only a few patients would still be living after this date and this was so (20 out of a total of 899 cases). A number of unpaired clinical cases and also of unpaired death certificates then remained. As regards the latter (76 males and 12 females), a special search revealed further clinical cases either in the disease index coded as 162 and 163, but missed in the first scrutiny, or in another part of the index or diagnosed as out-patients and therefore not appearing in the disease index. By these means all the death certificates were paired. A further search for pairs for the clinical cases revealed that 8 patients had died outside Leeds and therefore their death certificates were filed in another borough. All were correctly certified. Sixty-six clinical cases (54 males and 12 females) could not be paired with death certificates (Table VII). The basis for the clinical diagnosis was : without autopsy in 20 males and 2 females, by confirmatory autopsy in 11 males and 4 females, and by unexpected autopsy finding in 23 males and 6 females. The reasons for this discrepancy between clinical indexing and death certification are those stated above in relation to death certification, namely failure to amend the death certificate to fit the post mortem findings or ambiguous wording of the certificate.

TABLE VII.—*Comparison of Clinical Diagnosis with Death Certificates*

	Male	Female	Male	Female	Total No.	Total Per cent
Tally with death certificate	714	99	—	—	813	92·5
Including:						
Confirmatory autopsy	—	—	145	15	—	—
Not diagnosed in life, but revealed at autopsy	—	—	65	11	—	—
Notes lost but diagnosis known	—	—	10	1	—	—
Non-tally with death certificate	54	12	—	—	66	7.5
Total clinical cases	768	111	—	—	879	—
Still alive	17	3	—	—	20	—

Estimate of extent of agreement of clinical findings with death certificates

Excluding the 20 cases still alive at the end of the investigation, it is seen from Table VII that 813 of 879 cases (92·5 per cent) diagnosed in the hospitals of Leeds were certified as cancer of the lung at death. Sixty-six cases were either not certified or not coded as cancer of the lung, although 44 were submitted to autopsy and were proved cases, while 22 were judged clinically to be certainly due to this cause.

There are two causes for the failure of the clinical diagnosis to appear in the death certificate records: (1) the words carcinoma of the lung do not appear in any part of the death certificate (44 cases, Table VIII) and (2) they do appear but in a form or position which causes difficulty in coding (22 cases). In regard to the first group of cases, the tumour might be stated to be present in the mediastinum (code no. 164) or the certificate might be issued before the autopsy was done and not be amended afterwards, or death might be certified as due to another cause, no mention of cancer of the lung as a contributory cause being made. In regard to the second group of cases, some were certified by the Coroner, when it was the usual practice not to code any but the immediate cause of death and some were assigned to Part 2 of the certificate (Table VIII) and therefore would

TABLE VIII.—*Mode of Death Certification and Coding of 66 Clinically Diagnosed Cases of Cancer of the Lung not Certified as such*

Death certificates						Coded to			
Position of words "carcinoma of lung" on certificate						Respiratory disease		Other disease	
Autopsy			No autopsy			Cancer not specified as of lung or bronchus	Non-cancer	Cancer	Non-cancer
Part 1	Part 2	None	Part 1	Part 2	None				
6	2	14	6	8	30	8	14	21	23

never appear in code no. 162 or 163. Some certificates were worded in such a way that application of the coding rules demanded that another disease should take precedence over carcinoma of the lung in the allotted code number. For instance, in one certificate carcinoma of the bronchus appeared in part 1A but in part 1B an old tuberculous lesion of the lung appeared, to which disease the certificate was coded.

At this stage, inquiry from the Registrar-General was instituted to ascertain whether the coding of these 66 cases was similar to that adopted in Leeds. As the coding records for all years prior to 1954 had been destroyed, it was necessary to recode the certificates of all 66 cases and this was done by the method in use at the time and the method in current use. Two facts were ascertained: (1) in only 4 of 66 cases was there a difference of code number using the previous compared with the current method and (2) 7 cases were coded as carcinomas of the bronchus by the Registrar-General but had been coded by the Medical Officer of Health for Leeds in some other way. It thus seems probable that these 7 cases would form part of the 15 extra cases recorded by the Registrar-General (see p. 2).

TABLE IX.—*Estimate of the Degree of Accuracy of the Clinical Diagnosis of Cases in Two Hospitals*

Category	Means of diagnosis	Male	Female	Total No.	Total Per cent
I	Clinical histology	222	31	253	
	Confirmed by autopsy	156	19	175	
	Diagnosed by autopsy	88	17	105	
	Total	466	67	533	60·0
II	Ancillary methods without histology	301	44	345	38·8
III	Physical examination and history	9	2	11	1·2
	Total	776	113	889	

Estimate of the degree of validity of diagnosis of the clinical cases

In Table IX the clinical cases are classified according to international agreement. Omitting the 11 cases of which the notes were lost, it is seen that in 533 of 889 cases (60 per cent) a histological or autopsy diagnosis was made, in 345 (39 per cent) the diagnosis was made by ancillary methods and in only 11 (1·4 per cent) was it made by physical examination and history only. If 267 cases in class II, where the consultant gave the opinion that all the evidence (short of histological examination) was greatly in favour of a diagnosis of cancer of the bronchus, are added to class I, then a firm diagnosis was obtained in 92 per cent of cases.

DISCUSSION

If death certificates are to be used as a basis for the analysis of data relating to the properties of any disease, the clinical basis on which they are founded must be as accurate as possible. It was thought that in a provincial town such as Leeds, where special facilities exist for the diagnosis and treatment of intrathoracic cancer, it should be possible to trace the clinical records of those patients certified as having died of this disease and to form an estimate of the degree of validity of the clinical diagnosis in relation to death certification. This was done by a doctor experienced in tracing and scrutinising clinical notes and we consider that this is a very necessary part of the investigation, as it greatly encourages the co-operation of hospital records officers, departmental secretaries, general practitioners and others. The knowledge and experience of a doctor are also required in order

to interpret the notes. Search was continued until no other possible sources of information were left, with the result that only 37 of 1036 death certificates (3·6 per cent) were not traced (Table I).

Omitting these 37 certificates about which no clinical data could be obtained, it was found that the clinical diagnosis or autopsy record tallied with that recorded on the certificate in 964 of 999 cases, while in only 35 cases was the clinical diagnosis of another disease made in life and yet the certificate recorded death from carcinoma of the lung (Table I). The possible reasons for these discrepancies are discussed on p. 3, while the type of clinical diagnosis is shown in Table II. It is not difficult to understand why metastatic lung cancer (accounting for 23 cases) should occasionally be recorded as primary lung cancer, but it might be expected that non-cancerous conditions should not appear in this way.

In regard to the accuracy of the clinical diagnosis of cancer of the bronchus, the death certificates were traced back to the clinical notes in 953 cases (Table IV). In half of these, the diagnosis could be accepted as unequivocal (class I). In another 333 cases, the consultant in charge of the case was confident that the diagnosis was correct, leaving 143 cases (15 per cent) where the diagnosis was in considerable doubt. It is evident that where the clinical diagnosis was by no means certain (Table IX, class II) autopsy confirmed the condition in a large proportion of cases (Table V).

These findings are similar to those reported by McKenzie (1956) who used a different method of sampling. A questionary was sent from the General Register Office to the certifying medical practitioner in respect of every second death ascribed to cancer of the lung or bronchus in January, 1955. From 770 inquiries dispatched 654 replies were received. The validity of diagnosis was classified in much the same way as we have done and it was found that the standard of diagnostic technique was high : in only 3 per cent had no confirmatory procedure been adopted. Of a total of 634 certificates in only 18 cases was the certification of carcinoma of the lung not supported. In 8 of these the lung was found not to be carcinomatous and in the remaining 10 the lung condition was secondary to a primary elsewhere. It is interesting to note that in the 8 cases wrongly diagnosed as carcinoma the diagnosis was corrected in 4 by a post mortem examination made subsequent to the issue of the death certificate, and in the remaining 4, where necropsy apparently confirmed the diagnosis, subsequent histopathology disproved the macroscopic findings. The system obtaining in most hospitals, whereby the death certificate must be completed by the resident doctor before the post mortem examination is performed, leads in this way to a failure to amend the death certificate when the findings of the necropsy are known. It is always possible to overcome this difficulty if the resident doctor will initial Box B on the back of the certificate.

In addition to estimating the accuracy of the clincial data upon which the death certificates were based, an analysis was made of the mode of death certification of the cases diagnosed at the two main hospitals. Omitting the 20 patients known to be still alive, 813 of 879 cases diagnosed in the hospitals had death certificates which recorded the clinical diagnosis (8 of these were certified in another borough) and there were 66 in which cancer of the lung did not appear on the death certificate (7·5 per cent), or where the disease was not assigned to the coding carcinoma of the bronchus. In regard to the cases certified correctly, the clinical diagnosis was unequivocal in 60 per cent and highly probable in another 39 per cent (Table

IX). In the 66 cases certified as dying from other causes, the discrepancy was due either to the method of wording of the certificate or to difficulties in coding (see p. 8). Some of these discrepancies could have been avoided if the rules for certification and coding had been perfectly applied. But death certification does not and cannot be expected to give an exact picture of morbidity, as a few cases of cancer of the bronchus will be cured and others will die of independent causes, such as accidents and acute diseases.

An analysis of the clinical diagnosis in the 294 cases certified as dying of cancer of the lung and submitted to autopsy (Table V) is interesting. Where the autopsy was confirmatory of the clinical diagnosis, by far the largest group (76 per cent) had been diagnosed by ancillary methods only. In many of these cases there would either not be time to make a firm diagnosis or the consultant would consider that the evidence was strong, even in the absence of histological examination. Of the autopsies which revealed the condition for the first time, in one half another condition, either malignant or non-malignant, had been diagnosed and in the remaining half either no attempt at diagnosis had been made or the death was sudden. Of the 27 cases in which autopsy did not reveal cancer of the lung, but the death certificate recorded the condition, the diagnosis had been considered to be well established in 9 but in 17 there was no written clinical evidence for it and there was a real error in certification. This represents a false positive error in the death certificates of 9 per cent as revealed by autopsy, and this is largely contributed to by the 17 cases for which there was no written clinical evidence that the disease had ever been diagnosed in life. When the autopsy records of all the clinical cases in the two hospitals are analysed by Willis's method (Table IV) false positive diagnoses amount to 8 per cent.

The conclusion is drawn from all the above facts that the positive error in certification of cancer of the lung in Leeds is small (3·5 per cent). In fact, a larger number of cases which are diagnosed clinically fail to appear among the death certificates (7·5 per cent). The reasons for the errors are partly due to ambiguity of wording of the certificates, partly to mis-diagnosis and partly to the intervention of other causes of death.

It had been thought previously (Bonser and Thomas, 1955) that the discrepancy in certification was greater in females than in males and it was suggested that it was possible that more female deaths from metastatic lung cancer were recorded as primary lung cancer than were male deaths from this cause. No support could be obtained for this suggestion from this survey, 7 of 10 females and 16 of 25 males having been certified as dying from primary lung cancer when the disease was really metastatic (Table II). It was noted, however, that the female to male sex ratio was lower both for those cases incorrectly certified as lung cancer and for those positively diagnosed cases which were not certified as lung cancer. These effects tend to cancel one another out and there is no reason to suppose that the true sex ratio is materially different from that revealed by death certification.

CONCLUSIONS

1. 1036 death certificates recorded in Leeds city in the years 1950–54 and coded as cancer of the trachea, pleura, lungs or bronchi (*i.e.* code no. 162 and 163) were scrutinised. The clinical records of all but 37 were traced. It was found that 3·4 per cent of the remaining 999 cases were incorrectly certified and that the

remainder were certified on unequivocal evidence (50 per cent), probable evidence (45 per cent), and doubtful evidence (5 per cent).

2. The clinical notes of 879 similar cases recorded in the admission index of the two main hospitals in Leeds from 1950–54 were compared with their death certificates. 92·5 per cent were correctly certified at death and in 7·5 per cent the presence of the disease did not appear in the final coding by the Statistics department of the Medical Officer of Health for Leeds.

The reasons for the failure of cancer of the lung to appear in the coding might be: (*a*) the position of the words "cancer of the lung" on the certificate; (*b*) failure to record carcinoma of the lung on the certificate at all; or (*c*) the fact that the cancer of the lung was an incidental finding not causing death. More cases were diagnosed clinically and not certified at death, than were certified at death and not diagnosed clinically.

3. Analysis of the cases subjected to autopsy supported the finding that false positive certification was infrequent.

4. No sex difference in accuracy of certification was established.

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Addendum

Since the above communication went to press, an interesting comparison of diagnosis before and after post mortem has been made by the Registrar-General, who arranged for death certificates to be completed by a clinician immediately after death and by a pathologist immediately after post mortem in 1,404 cases in 10 hospitals in England. These represented 81·2 per cent of all deaths in these hospitals for the period under review, There was agreement in 51 per cent of cases, while in another 28 per cent the disagreement was one of opinion rather than of fact. Cancer of the lung was notably under-diagnosed by clinicians, the amount of error being greatest over the age of 65 years.

Reference.

Registrar-General's Statistical Review of England and Wales, 1956. Part III. Commentary, p. 182.

CANCER OF THE MOUTH: 11-YEAR FOLLOW-UP OF 800 CASES

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In 1950 Ledlie and Harmer published a report on 800 patients with cancer of the mouth seen at the Royal Marsden Hospital (then the Royal Cancer Hospital) between January, 1936, and December, 1945, with a minimum follow-up period of 3 years. These same patients have been reviewed again up to the end of December, 1956, the minimum follow-up period being 11 years. The success or failure of the first treatment is reassessed in terms of recurrences.

GENERAL REVIEW OF 800 CASES

In the 10 year period 1936–1945 inclusive 800 new cases were seen and these were grouped into 9 sites. The number of cases included in each site is shown in Table I and the number of survivors at 5 and 11 years is indicated in the second and third columns of this Table.

TABLE I.—*The Cases by Sites*

Primary sites	Number of new cases, 1936–45	Number of survivals, minimum 5 years	Number of survivals, minimum 11 years
1. Mucosal surface of lips	160	59	28
2. Mucosa of upper alveolus and hard palate	40	10	5
3. Mucosa of lower alveolus	54	8	4
4. Buccal mucosa	59	11	1
5. Floor of mouth and inferior surface of tongue	136	18	7
6. Dorsum and borders of anterior two-thirds tongue	150	25	10
7. Posterior third tongue (and whole tongue)	75	6	2
8. Anterior faucial pillar and soft palate	85	6	4
9. Tonsil proper	41	6	4
	800	149=18·8%	65=8.1%

The age groups, duration of symptoms before treatment, staging, histology and multiplicity of primary tumours were discussed in the previous paper (Ledlie and Harmer, 1950) and the results of the various treatments employed were evaluated. In this present paper it is intended to analyse further those patients who were still surviving in December 1950, giving a minimum 5-year follow-up of all cases in the previous paper, and to correlate the survival time with the staging, histology, first treatment and subsequent treatments of each case individually. In order to simplify the tables in the previous paper (1950) the primary

sites were condensed into 4 groups and the number of cases in each group at that time is indicated in Table II. The right hand column of Table II shows the number of cases considered in this paper, namely 149.

TABLE II.—*Primary Sites in 4 Groups*

	Number of cases in original paper		Number of cases in present paper (5-year survivals)	
Lip	160		59 (Table II)	
Anterior two-thirds of tongue	150		25 (Table IV)	
Other buccal sites:				
Upper alveolus and hard palate	40		10	
Lower alveolus	54	289	8	47 (Table V)
Buccal mucosa	59		11	
Inferior surface of tongue and floor of mouth	136		18	
Oro-pharyngeal sites:				
Posterior one-third tongue	75		6	
Anterior faucial pillar and soft palate	85	201	6	18 (Table VI)
Tonsil	41		6	
	800 cases		149 cases	

Multiple Primaries

The incidence of second primary tumours in Ledlie and Harmer's paper was 3·75 per cent, i.e., 30 patients in 800 cases. The majority of the new primary tumours occurred in the digestive tract and most often between the lip and cardiac sphincter of the stomach. In the present paper 12 patients out of the 149 who have survived since 1950 developed a new primary tumour, an incidence of 8 per cent. This suggests that patients with an oropharyngeal carcinoma are particularly liable to develop a further primary tumour if they survive long enough.

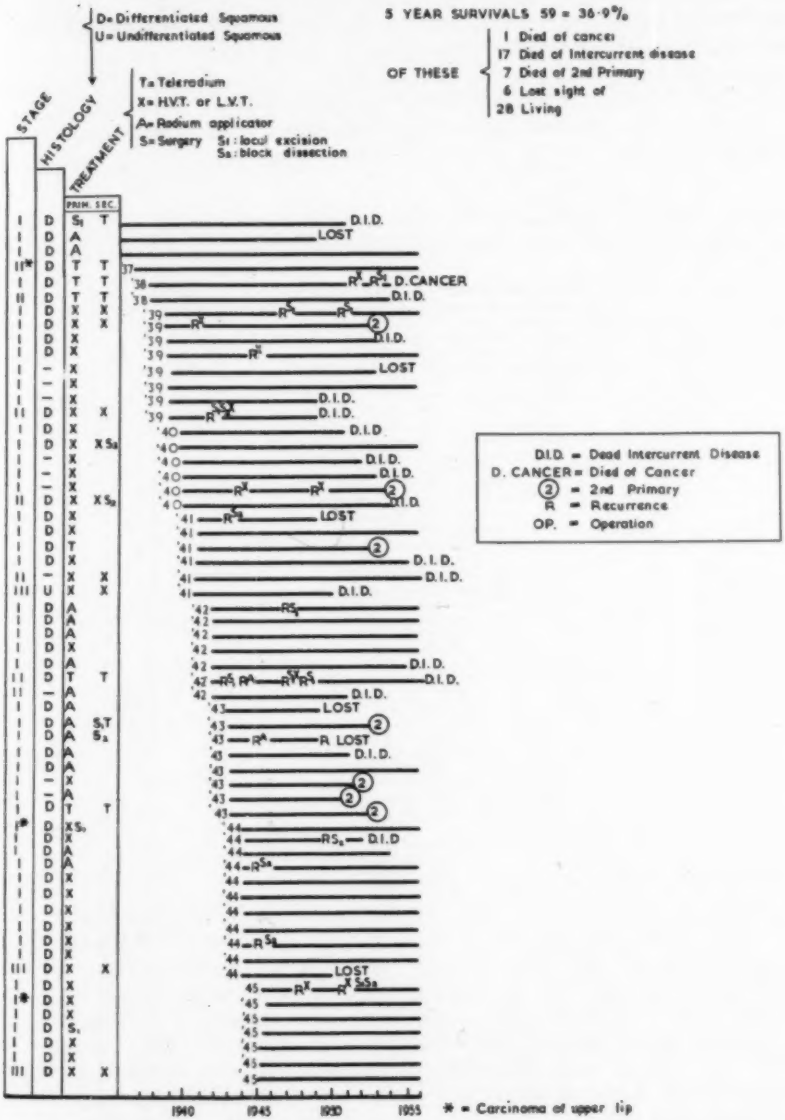
Mucosa of Lip (Table III)

The interesting feature of this group is that only one patient of the 59 considered died of his disease (a Stage I case who recurred after 13 years and who died 2 years later). Fourteen patients, however, had a recurrence of their tumour treated at one time or another. The good results for carcinoma of the lip may be attributed not only to its natural history but also to its accessibility for treatment by radiotherapy or surgery and to regular follow-up attendance. There was a high incidence of death from a new primary tumour elsewhere in the body, 7 cases occurring in this group—3 in the colon, 2 in the stomach and one each in the breast and bronchus.

In Stage I local excision of the primary growth was only employed on three occasions and these patients are recurrence-free after 14, 11 and 10 years respectively. Fifteen patients were treated by radium applicator and three of these recurred—two at the primary site and one in the cervical lymph nodes. These recurrences were treated by excision, radium needle implantation and block dissection of the neck respectively. These patients are alive and well 14, 12 and 6 years after first treatment.

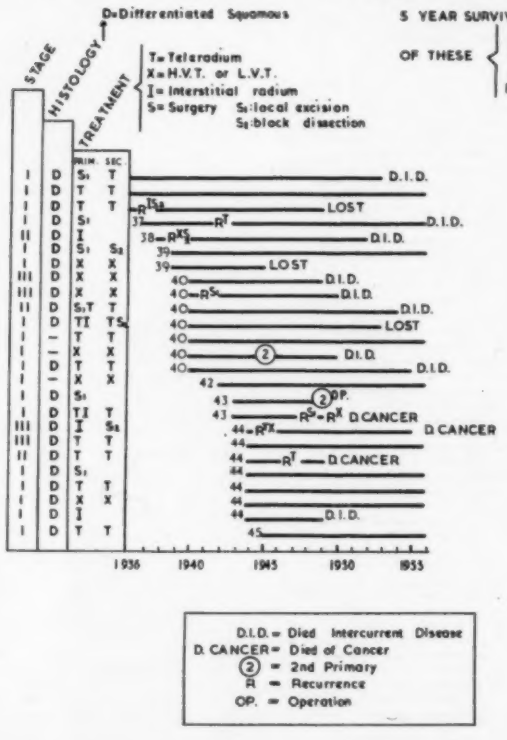
Forty-one patients were treated by external irradiation (superficial X-ray therapy 35: teleradium 6) and the results have been good, only one patient dying of recurrence after 13 years. Seven Stage II and 3 Stage III tumours

TABLE III.—*Mucosa of lip.* 1936–1945. 160 *Cases*



were treated by external therapy, one Stage II case also having a block dissection. None of these patients has died of cancer. External irradiation is very effective in the treatment of carcinoma of the lip in all stages, though wide local excision may be equally effective in Stage I cases. Radium therapy has given disappointing results in terms of local recurrence.

TABLE IV.—*Anterior two thirds Tongue.* 1936–1945. 150 *Cases*



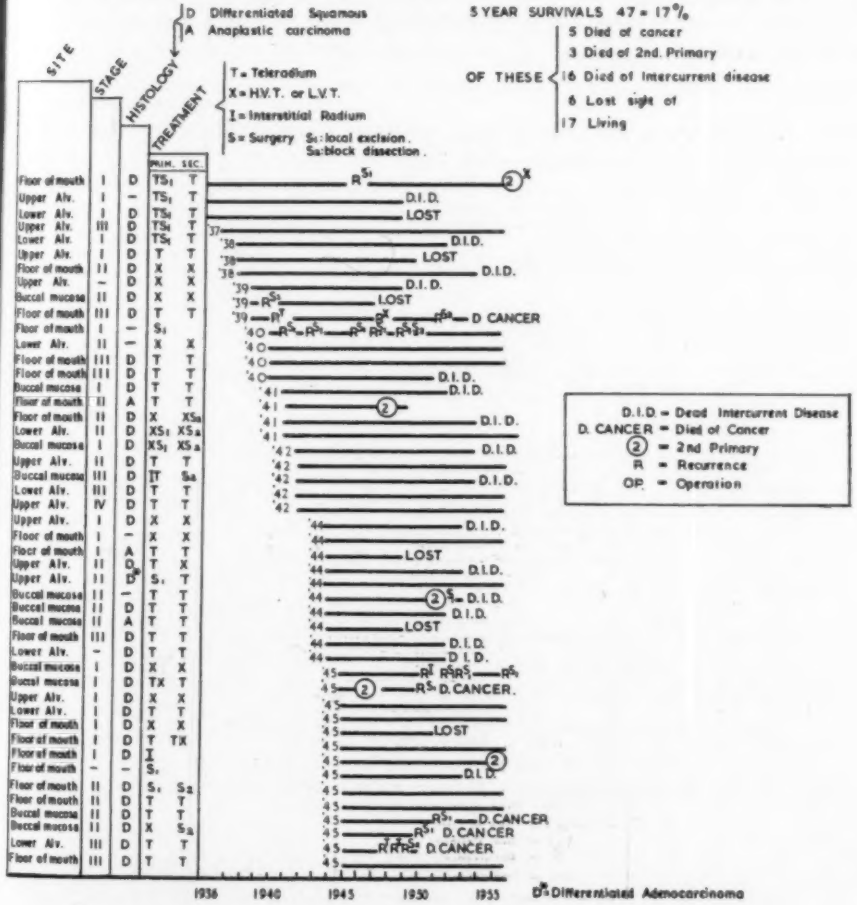
Anterior Two-thirds tongue (*Table IV*)

Interstitial radium is employed frequently in the treatment of cancer at this site. None of the patients still living, however, were treated by this method and 2 of the 3 cases who died of cancer received interstitial therapy in the first instance, combined in one case with teleradium and in another with a block dissection of the neck. Seven of the 9 patients still living were treated by external irradiation, the other 2 (Stage I cases) having a local excision, combined with a block dissection of the neck in one case. A wide local excision can cure the primary disease; teleradium and X-ray therapy gave good results and proved more effective than interstitial irradiation in this series, although the results of the latter treatment in some other centres do not confirm this (*vide* Table XV of Ledlie and Harmer's paper, 1950).

Upper Alveolus, Hard Palate, Lower Alveolus, Buccal Mucosa, Floor of Mouth and inferior Surface of Tongue (Table V)

Five patients in this group died of their disease and all were treated with external irradiation (teleradium 4 : X-ray therapy 1), 2 also having a block dissection of the neck. Only one of these, however, was Stage I when first seen. It is interesting to note that 3 patients in this group died of a new primary tumour in an area remote from the treated cancer, namely, bronchus, stomach and colon. Two of the cases demonstrate well the importance of regular follow-up for all cancer patients and these patients owe their survival to the combined vigilance

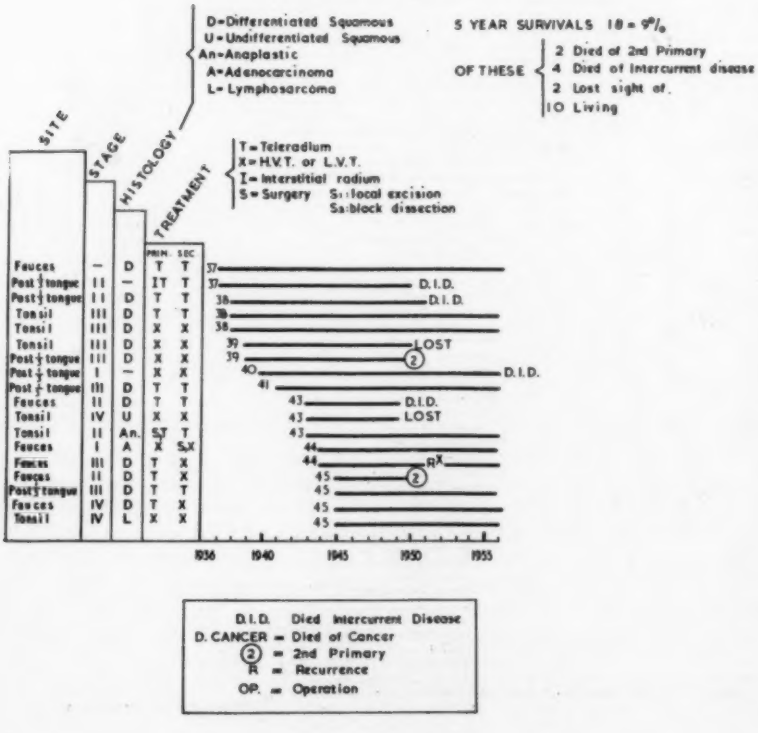
TABLE V.—*Upper Alveolus and Hard Palate, Lower Alveolus, Buccal Mucosa, Floor of Mouth and Inferior Surface of Tongue.* 1936–1945. 289 *Cases*



of the surgeon and radiotherapist. One case in particular had five local excisions and finally a block dissection and has now been recurrence-free for 7 years.

Seventeen patients in this group are still living and of these 12 had external irradiation (teleradium 8 : X-ray therapy 4), 3 were treated by combined methods i.e., local excision and X-ray therapy, and 2 had a local excision and block dissection of neck. Interstitial therapy was only employed twice for the primary lesion, both of these patients surviving more than 5 years without recurrence. Combined methods of treatment have been preferred in Stage II and III cases as opposed to surgery alone. One half of the patients still living after external irradiation only were in Stages II, III or IV. Some of these patients would be regarded as surgically incurable and the results of radiotherapy are encouraging. To summarize : most of the cases in Stage II and III who are still living were originally treated by external irradiation (teleradium in almost all cases). It should be stressed, however, that 4 out of 5 cancer deaths were in Stages II and III and were treated primarily by radiotherapy, combined with a block dissection of the neck on one occasion.

TABLE VI.—*Posterior one third Tongue, Anterior Faucial Pillar, Soft Palate and Tonsil.* 1936–1945. 201 *Cases*



Posterior One-Third Tongue, Anterior Faucial Pillar, Soft Palate and Tonsil Proper (Table VI)

There are two striking features in this group, firstly the high incidence of survivors in Stages II, III and IV, only 2 being in Stage I, with one patient unstaged, and secondly that only one patient recurred later than 5 years after first treatment. All the 18 cases considered were treated with external irradiation (teleradium 11 : X-ray therapy 7), one case also having a local excision and another interstitial irradiation. None of this group eventually died of the disease ; 2 died of a new primary growth elsewhere. Histologically 12 out of the 18 cases had well-differentiated squamous cell carcinoma. Radiotherapy is the treatment of choice for all cases in this group, but nevertheless, only 9 per cent of patients treated survived 5 years.

CONCLUSIONS

The conclusions made by Ledlie and Harmer in their 1950 paper were :—

(1) Although very different methods of treatment may be employed the results are much the same.

(2) Carcinoma of the lip is best treated by superficial X-ray therapy.

(3) Small Stage I growths in accessible situations within the mouth may be widely excised, but can be treated with equal success by radiotherapy. Regular follow-up is particularly essential in these cases.

(4) Recurrence at the primary site is more common than is usually appreciated and may be as frequent as recurrence in the nodes after both have been apparently successfully treated.

(5) In most buccal and oro-pharyngeal sites the results of teleradium therapy are superior to those of X-ray therapy.

(6) The results of teleradium treatment for growths of the anterior two-thirds of the tongue has not been so successful as interstitial methods reported from other centres.

These conclusions were formulated on a minimum 3-year follow-up and remain largely true in the light of this more recent survey with a minimum 11-year follow-up period. Point (6) in the conclusions, however, has not been re-examined. Between 9 and 12 per cent of patients were lost to follow-up in the whole series. This figure would probably be improved upon at the present time, but many cases failed to attend during the war years, 1939–1945, and were consequently lost for follow-up purposes.

The conclusion of the original paper, that for treatment to be successful it must be radical in the first instance is supported and in addition it is confirmed that the only method of ascertaining complete eradication of the disease is regular out-patient follow-up.

I wish to acknowledge my debt to the staff of the Royal Marsden Hospital for permission to publish their clinical material. I am grateful for help from Miss June Akister who constructed the Tables and Miss Susan Taylor of the Records Department.

REFERENCE

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A COMPARISON OF TREATED AND UNTREATED CASES OF CANCER OF THE BREAST

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Whether or not treatment of cancer of the breast materially alters the survival rate is a problem which has given rise in recent years to much speculation and several papers in the medical literature. The evidence for treatment has been indirect, being based upon comparisons with groups of untreated patients not directly comparable with the treated groups. From a statistical point of view the best method of attacking the problem would be to treat alternate patients but for numerous reasons this is impossible. Another method, statistically acceptable, is to match each untreated patient with a treated one. Such a matching technique has been employed in the present paper.

This study includes 230 cases of untreated cancer of the breast which were collected from cancer treatment centres in Canada, England, France and the United States. For each case the following data were requested :—

(*a*) age at diagnosis
(*b*) duration of symptoms before diagnosis
(*c*) pathological diagnosis
(*d*) clinical findings
  (1) confined to breast (operable)
  (2) confined to breast and axilla (operable)
  (3) confined to breast or breast and axilla but inoperable because of fixation to chest wall or fixation of axillary lymph nodes
  (4) evidence of secondary deposits other than same breast or axilla
(*e*) reasons for no treatment
  (1) patient refused
  (2) patient too old
  (3) intercurrent disease
  (4) disease too advanced
(*f*) survival, from diagnosis.

Table I shows the age distribution of the 230 untreated cases compared with two treated series, one of 783 cases from the Toronto General Hospital in Ontario (Phillips, 1954, unpublished study) and one of 993 cases from the province of Saskatchewan (Watson, 1951). It will be noted that approximately 60 per cent of the untreated cases are over 60 years of age with an average age of 64 years. This average age is high compared to the two treated groups, illustrating one of the problems in comparing series of cases.

Data on duration of symptoms were given for 181 of the untreated cases. The average for these 181 cases was 27·1 months with the longest being 204 months and the shortest one month.

TABLE I.—*Age Distribution of Untreated and Treated Cases of Cancer of the Breast*

Age group	Proportion in each age group		
	Untreated (%)	Ontario (%)	Saskatchewan (%)
Under 30	0·4	1·4	1·9
30–39	4·4	8·6	10·1
40–49	12·2	22·0	26·1
50–59	20·2	26·2	33·5
60–69	25·4	24·4	19·2
70–79	25·2	14·1	8·1
80 plus	12·2	3·3	1·1
Average age	64 years	56·3 years	52 years

Table II shows the extent of the disease in the untreated group as determined by clinical examination. It will be noted that the 84 cases in whom the disease was confined to the breast and to the breast and axilla have been classified as "operable". These cases have received further study later in this report.

TABLE II.—*Extent of Disease as Determined by Clinical Examination in Cases of Untreated Breast Cancer*

Extent of disease	Number of cases	Per cent
Confined to breast (operable)	43	18·7
Confined to breast and axilla (operable)	41	17·8
Fixation to chest wall or of axillary lymph nodes	32	13·9
Secondary deposits of disease	106	46·1
Not specified	8	3·5
	230	

Pathological proof of malignancy was obtained on 46 cases or 20 per cent of the untreated series. Of the remaining 184 cases, a biopsy was not taken in 176 and in 8 information on this point was not given.

Table III shows the various reasons why these patients were not treated and it will be noted that the two main reasons were that the disease was too advanced or the patient refused treatment.

TABLE III.—*Reasons for not Treating Cases of Untreated Breast Cancer*

Reasons	Number of cases	Per cent
Patient refused	84	36·5
Patient too old	19	8·3
Intercurrent disease	5	2·2
Disease too advanced	101	43·9
Other	11	4·8
Not specified	10	4·3
	230	

Two measures of survival have been calculated for this group of untreated patients. The first of these is the survival from the date of examination or diagnosis

and the second from the date of alleged onset of symptoms. These data are shown in Table IV.

TABLE IV.—*Survival Calculated from Date of Diagnosis and Date of Onset of Symptoms for Untreated Breast Cancer Cases*

Survival from	Number of cases	Mean survival (months)	Survival (%)					
			1st yr.	2nd yr.	3rd yr.	4th yr.	5th yr.	10th yr.
Date of diagnosis	230	19·2	37·0	22·5	15·0	11·5	9·5	1·3
Onset of symptoms	181*	46·2	75·2	58·2	44·0	34·0	28·5	6·6

* Data on duration of symptoms available on 181 cases only.

The average duration of life from onset of symptoms, 46·2 months, compares with 40·5 months quoted by Daland (1927), 39·8 months quoted by Lazarus-Barlow (1924), 39·6 months quoted by Wyard (1925) and 38·0 months quoted by Wade (1946).

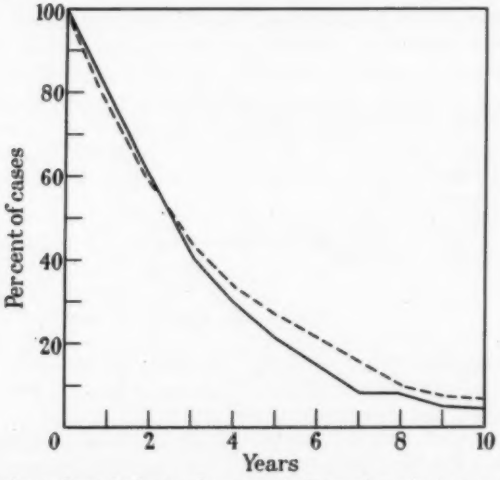

FIG. 1.—Comparison of survival rates from onset of symptoms in two series of untreated breast cancer patients. ———— Daland (1927). – – – – Present series.

In Fig. 1 the annual survival rates from onset of symptoms have been compared with those reported by Daland (1927) and it will be noted that the two groups of cases show approximately identical trends.

Comparison with Treated Patients

The relative value of treatment in breast cancer has been assessed by matching each untreated operable case with a treated one from the Toronto General Hospital. Patients were matched on age at diagnosis and on age at alleged onset of symptoms and a variation of one year was permitted. Since many treated patients were of the same age as the untreated one, the selection of the treated patient was made by using the table of random numbers. The extent of disease in the matching

process was controlled since operable cases in the untreated series were matched with Stages I and II in the treated group where :—

STAGE I is described as the tumour localized in the breast, skin not involved and no metastases evident in the axillary lymph nodes or elsewhere.

STAGE II is described as the tumour localized in the breast, moveable, skin not involved and axillary lymph nodes involved but no other evidence of metastases.

In the untreated series 84 cases were classified as operable and these have been matched with 84 treated cases on the basis of age at diagnosis. The duration of symptoms was not given on 19 of the untreated operable cases hence the matching on the basis of age at alleged onset of symptoms was made on 65 cases. For those patients matched on age at diagnosis the survival rates have been calculated from date of diagnosis and are shown in Table V and Fig. 2. The differences

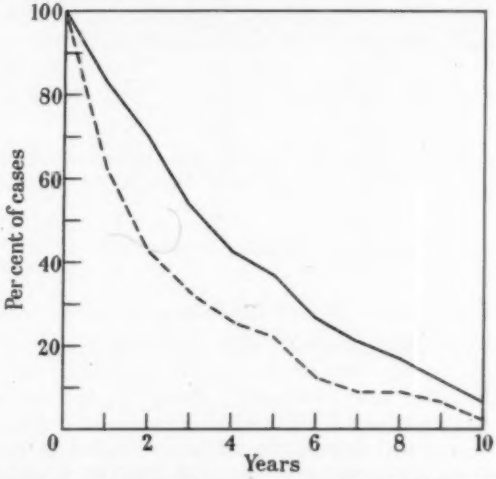

FIG. 2.—Survival rates from date of diagnosis for treated and untreated patients matched for age at diagnosis and extent of disease. ———— Treated. - - - - Untreated.

TABLE V.—*Comparison of Survival Rates from Date of Diagnosis for Treated and Untreated Patients Matched for Age at Diagnosis and Extent of Disease*

Time (years)	Untreated ($N = 84$) Number surviving	Per cent	Treated ($N = 84$) Number surviving	Per cent	Difference
1	53	63·1	70	83·3	Significant*
2	36	42·8	59	70·2	"
3	28	33·3	45	53·6	"
4	22	26·2	36	42·8	"
5	19	22·6	31	36·9	"
6	11	13·1	23	27·4	"
7	8	9·5	18	21·4	"
8	8	9·5	14	16·7	Not significant
9	6	7·1	10	11·9	" "
10	2	2·4	6	7·1	" "

* At the 0·05 level of confidence.

between the proportions of patients surviving at each year have been tested for significance by the chi-square technique. It will be noted that the proportion of treated patients surviving is significantly greater than untreated at each year to eight years.

The survival rates for untreated and treated cases matched for age at onset of symptoms have been calculated from the date of onset of symptoms and are shown in Table VI. The significance of the differences in the proportion surviving at each year have been tested by the chi-quare method. It will be noted that the differences are not significant except at the two-year point.

TABLE VI.—*Comparison of Survival Rates from Onset of Symptoms for Treated and Untreated Patients Matched for Age at Onset of Symptoms and Extent of Disease*

Time (years)	Untreated ($N = 65$) Number surviving	Untreated ($N = 65$) Per cent	Treated ($N = 65$) Number surviving	Treated ($N = 65$) Per cent	Difference
1	54	83·1	60	92·3	Not significant*
2	45	69·2	57	87·7	Significant
3	43	66·1	48	73·8	Not significant
4	33	50·8	39	60·0	" "
5	29	44·6	30	46·1	" "
6	22	33·8	23	35·4	" "
7	14	21·5	20	30·8	" "
8	9	13·8	18	27·7	" "
9	8	12·3	14	21·5	" "
10	6	9·2	12	18·5	" "

* At the 0·05 level of confidence.

DISCUSSION

The purpose of studying untreated cases of breast cancer is two-fold since it provides information on the natural history of the disease as well as an opportunity to assess the efficacy of treatment. However, the number of such cases is limited since patients who do not receive surgery or radiation therapy often receive hormone therapy and therefore must be considered as having been treated. Further, those who are untreated are often not followed hence the survival time is unknown. With these limiting factors it is felt that the present series of 230 cases constitutes a substantial sample of this type of cancer patient.

Since the average age of this untreated group is considerably greater than that of treated groups one is not justified in comparing the untreated series with a treated one. To overcome this problem and to ensure that some measure of similarity exists between the two groups it becomes necessary to consider matching patients. In the matching process three variables have been considered, age at diagnosis, extent of disease and duration of symptoms as determined by age at alleged onset of symptoms. The validity of data on alleged duration of symptoms has been questioned repeatedly. In the untreated cases being studied the average duration of symptoms was 27·1 months which is considerably greater than that reported in treated series and the lack of significance in the survival from onset of symptoms between treated and untreated patients may be due to inconsistencies in these data.

Another factor which affects the matching of untreated and treated patients is the proportion of cases in each group having pathological proof of malignancy.

In most treated series the proportion is approximately 75 per cent and it is probably closer to 100 per cent in the matched treated patients being considered in this paper. On the other hand only 17·5 per cent of the matched untreated cases were diagnosed pathologically. This weighting of untreated cases on the side of clinical diagnosis as opposed to microscopic could favour the untreated group by including lesions which would be diagnosed microscopically as benign.

SUMMARY

A study has been made of 230 cases of untreated breast cancer.

The natural history of the disease as revealed by these cases has been compared with an earlier series by Daland (1927).

The average age and average duration of symptoms for the untreated cases were greater than those reported for treated cases.

Untreated operable patients have been matched with treated operable patients from the Toronto General Hospital, Ontario, on the basis of age at diagnosis and age at onset of symptoms.

In comparing treated and untreated patients, matched for age at diagnosis it was found that :—

(*a*) the survival rate calculated from date of diagnosis was significantly greater for treated patients than for untreated ones for each year to eight years.

In comparing treated and untreated patients, matched for age at onset of symptoms, it was found that :—

(*b*) the survival rate calculated from onset of symptoms was consistently higher for treated patients than for untreated ones but the differences were not significant.

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EARLY DIAGNOSIS OF MALIGNANT CONDITIONS IN LYMPH NODES

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The diagnosis of a malignant condition is always of very serious significance. If the diagnosis is correct, the future life of the patient is troubled and the ultimate fate is dark.

But, even if the diagnosis is incorrect, it is accompanied by grave consequences. The patient is stigmatized, he lives in a tense condition full of anxiety, and he is likely to be subject to radical therapeutic steps, to surgery or intensive irradiation, not seldom followed by a more or less marked reduction of his activity and well-being.

In our days the situation is aggravated by the intensive propaganda for " early diagnosis ", as well as by the therapeutic perfectionism aiming at " cure ". The plea for early diagnosis by its own momentum leads to a diagnosis supported by small deviations from the normal, small in quality and small in quantity. The " earlier " the changes, the greater the uncertainty.

This problem often is presented :— which are the therapeutic steps to be taken on the basis of uncertain clinical and histological data ? In few fields is this uncertainty better felt than when lymphoid tissue is involved.

In the present paper an analysis will be made of a series of cases, where the clinician, in the presence of enlarged lymph nodes, decided to have a biopsy examined—and where, after the examination, the pathologist gave a diagnosis of :— " Lymphoid hyperplasia. Malignancy cannot be excluded ".

The material is part of a total of 3483 lymph nodes received at our laboratory in the years 1946–55. Out of this series 124 cases were found, presenting the criteria mentioned.

The primary diagnosis in the following is the designation for the original diagnosis, with the accompanying remark. These diagnoses were, during a period of 10 years, given by 5 different pathologists. The diagnostic criteria, the descriptions and the terminology are accordingly not uniform.

The 124 cases can, corresponding to the terms and expressions used, be divided into two grades of uncertainty. Grade II means that the suspicion of malignancy was moderate. Grade III means that the suspicion was grave.

This material was now made the subject of a *revised diagnosis*, performed by one pathologist (K), according to uniform criteria and without any knowledge of the clinical information or of the previous (primary) diagnosis.

Also this material was now " graded ", with Grade I meaning that the morphological criteria does not substantiate a diagnosis of malignancy. Grade IV means an unreserved diagnosis of malignancy. Also this material will have Grades II and III, indicating a slighter or a graver suspicion of malignancy. These Grades II and III are not based upon the same criteria as those of the primary diagnosis, but, it is nevertheless regarded as useful to correlate these grades.

The patients were clinically re-examined and their fates registered. In every

case satisfactory information was obtained. Very helpful, as usual, was our Cancer Registry. We are likewise indebted to the clinicians who generously gave us their help.

More than $\frac{2}{3}$ of the cases (86) had an observation period of 5 years, or more, and all cases had an observation period of 3 years or more.

As our study revealed that the patients dying from, or with, a malignant lymphoid disease had an average survival time of 2·57 years and a median survival time of 1·25 years after the biopsy, we regard a limit of 3 years as acceptable.

TABLE I.—*The Result of the Clinical Follow-up*

Clinical development	Number		
	M.	F.	Total
Symptom-free	29	20	49
Died from intercurrent disease	2	3	5
Suffering from malignant lymphoid condition	10	5	15
Died from or with malignant lymphoid condition	39	16	55

TABLE II.—*The Final Clinical Diagnosis in Cases of an Accepted Malignant Condition*

	Died			Alive		
	M.	F.	Total	M.	F.	Total
Lymphoid leukaemia	18	6	24	2	1	3
Lymphosarcoma	16	7	23	5	3	8
Giant follicular hyperplasia	1	0	1	0	0	0
Reticulosarcoma	1	2	3	1	0	1
Hodgkin's disease	3	1	4	2	1	3

Correlation of primary histological diagnosis and final clinical development

As already mentioned, during the primary diagnostic work, no grading was performed. A grading was, however, done during the present study and based upon the original phrasing of the suspicion of malignancy.

Grade II, representing cases with a moderately worded suspicion of malignancy, embraces 41 patients. Two patients died from an intercurrent disease 1 and 11 years after the biopsy was made. Of the remaining 39 patients 7 (18 per cent) developed a malignant lymphoid condition, the others living symptom free.

It may be mentioned that every one of these 7 malignant cases was correctly diagnosed during the revision. This indicates that the histological material actually was representative. The suspicion of malignancy was substantiated in 18 per cent of the cases only.

When 34 patients were under a certain suspicion of malignancy after examination of the morphological material, in spite of the later benign clinical course, this indicates that the diagnosis had been built upon very small deviations from the normal, and that the pathologists involved probably had been influenced either by the case history or by a philosophy of aggravating the diagnosis for "safety sake".

Grade III, representing cases with a more strongly worded suspicion of malignancy, embraces 83 patients. Three patients died from intercurrent disease $\frac{1}{2}$, $\frac{1}{2}$ and 1 year after the biopsy. Out of the remaining 80 cases a malignant lymphoid condition was diagnosed in 63, that is in $\frac{4}{5}$ of the cases. Among the 17 symptom free, there are 5 patients with only 3 years of observation, so that a few more may develop malignancy. Two only, of the 17 living symptom free, were

given specific treatment. A postulated " cure " can therefore not materially alter the evaluation.

Correlation of the revised histological diagnosis and the final clinical development

Grade I represents cases where, during revision, the morphological findings did not substantiate a diagnosis of malignancy, a total of 40 cases. Four patients died from intercurrent disease ½, 1, 1, and 11 years after the biopsy. Among the remaining 36 cases 2 only showed development of a malignant lymphoid condition (5 per cent). Both these cases were from the primary diagnosis classified as Grade III.

Case 1. A 14 years old boy, with marked clinical symptoms mentioned in the case history submitted to the pathologist. The slide has been restudied several times, but we cannot find a sufficient morphological basis for a diagnosis of malignancy. Most probably the diagnosis was made on non-representative material and a suggestive case history. The lymph nodes had been irradiated.

Case 2. A 61 years old man dying of lymphatic leukaemia within one year after the biopsy. The slide has been re-examined several times and is regarded as difficult to interpret owing to a considerable admixture of plasma cells and eosinophiles. In this case a diagnostic failure must be admitted.

Grade II embraces only 4 patients, 2 living symptom free, 2 developing a malignant lymphoid condition.

In both cases of malignancy, the lymph nodes were small, the changes minor, but in both cases lymphocytic infiltration of the capsule was present. These two cases should have been referred to Grade III.

Grade III embraces 24 patients out of which 19 (¾) showed a malignant lymphoid condition, whereas 5 patients remained symptom free in the period of observation 10, 8, 7, 5 and 3 years after the biopsy.

In the slides from these patients four cases gave the picture of a well conserved architecture of the lymph nodes, but the follicles were considerably enlarged and showed a great number of mitoses. This finding will be commented upon later.

In the fifth case the histological picture was very like that of the case in Grade II where leukaemia developed later. Here again the criteria did not permit a correct diagnosis.

Grade IV, representing cases where the malignancy diagnosis was now given unreservedly, embraces 56 cases, with one patient dying from an intercurrent disease ½ year after the biopsy. Of the 55 remaining, 47 showed a malignant lymphoid condition, whereas 8 remained symptom free. One of the latter suffered from infectious mononucleosis, which means a definite complicating factor for a correct diagnosis. In the other cases the histological picture was still after several revisions regarded as being of a grave character. The observation periods were one case 11 years, two cases 8 years, one case 7 years, one case 4 years, and two cases 3 years.

TABLE III.—*Results of Histological Grading*

Malignancy grade	Series: Primary		Series: Revised	
I	—		40	
II	41	124	4	28
III	83		24	
IV	—		56	

The first striking result of the revision is that from the 124 doubtful diagnoses 28 only (22·5 per cent) are left as such. The others have been placed with diagnoses with or without the affix malignancy, without a question mark. This means that the revised diagnosis through its pertinent criteria is more categorical. The differentiation is greater. But, nothing is thereby implied as to the correctness of the diagnosis. The relative merits of the two diagnostic series will have to be further analysed.

TABLE IV.—*Correlation of the Primary and the Revised Histological Diagnosis and Clinical Development*

	Malignancy grade	Symptom free (a)	With malignant lymph. manifest. (b)	Ratio a : b	
Primary diagnosis	II	32	7	5 : 1	
	III	17	63	1 : 4	
Revised diagnosis	I	34	2	17 : 1	
	II	2 } 7	2 } 21	1 : 1	1 : 3
	III	5 }	19 }	1 : 4	
	IV	8	47	1 : 6	

The five patients dying from intercurrent diseases have not been included.

The most important findings are :

(i) The revision of the doubtful cases from the primary diagnostic series has led to a reduction of doubt to one out of five cases only.

(ii) The new categorical formulation of the diagnosis in the previous doubtful cases has resulted in a fair accuracy :

(1) if the diagnosis is given as benign, there is a 17 to 1 chance that this is correct,

(2) if the diagnosis is given as malignant, there is a 6 : 1 chance that this is correct,

(3) where doubt is still entertained, there is a considerable chance (1 : 1 Grade II, 4 : 1 Grade III, average 1 : 3) that the condition under diagnosis is malignant.

These figures, taken with the limitation of the number of cases, give a measure of the accuracy of our diagnosis in such cases.

Before we discuss the implications of these facts, a short survey is given of the criteria found to be of greatest value in forming our diagnosis of the material under discussion.

Evaluation of the morphological criteria

In Table V the histological features are arranged in relation to the clinical development. The features are listed in such a manner, that those showing the greatest correlation with benign lymphoid conditions are on the top, and those connected with malignancy at the bottom of the table. In order to ease the comparison the occurrence is given in per cent.

Based upon these figures, a relative occurrence between the benign and the malignant conditions of each of the features examined has been calculated. If, for instance, " dominance of small lymphocytes " occur among the symptom-free

TABLE V.—*Histological Criteria and Diagnostic Significance in Regard to Benign and Malignant Lymphoid Conditions*

Criterion	Non-malignant cases	Value in favour of benignancy	Patients with malignant lymphoid conditions	Value in favour of malignancy
No capsular, or pericapsular infiltration	**56**	**95**	3	5
Architecture conserved	**74**	**74**	26	26
Dominance of small lymphocytes	**76**	**72**	29	28
Reticulum cell proliferation	36	65	19	35
Polymorph cell population	24	60	16	40
Fibrosis	26	58	19	42
Large follicles	24	55	20	45
Dominance of medium size lymphocytes	20	36	36	64
Increased number of mitoses	10	32	21	68
Capsular, or pericapsular infiltration	42	30	97	70
Obliteration of sinus	22	23	**74**	**77**
Architecture destroyed	10	19	**44**	**81**
Dominance of lymphoblasts	2	5	**36**	**95**

patients 76 times and among the patients suffering from a malignant lymphoid condition 29 times, this means that the relative occurrence in benign cases is $\frac{76 \times 100}{105}$, that is 72 per cent.

As would be expected, the commonest features in the benign conditions are those regarded as belonging to the normal lymph node. As likewise would be expected from the very selection of cases, no case presented only normal features.

The most important features pointing in the direction of a benign condition in the lymph node are: lack of infiltration of the capsule and the pericapsular tissue, conservation of the general architecture and dominance of small lymphocytes.

The findings indicate that if two, or especially three of these features are present, there is a very high probability that the patient is not suffering from a malignant lymphoid condition—of course with the proviso that the biopsy is representative. A striking illustration of the relevancy of this reservation is shown in Fig. 1, where a group of small lymph nodes was received for examination and one only out of the 12 showed malignant changes. All the others were normal.

The most important feature pointing in the direction of a malignant condition in the lymph nodes are: dominance of lymphoblasts, non-conservation of the general architecture and obliteration of the peripheral sinus.

The presence of two or all three of these features gives a very high probability of malignancy.

As regards the other features, none of them reaches sufficiently high in importance to be of very great diagnostic value.

As previously mentioned, one combination ought to be stressed, as already done by Rappaport, Winther and Hicks (1956) namely, large follicles and a great number of mitoses. Our findings completely agree that this combination is more linked with a benign condition than with giant follicular hyperplasia. We were not sufficiently aware of this situation, hence our classification of these as cases of malignancy (Grade III).

FIG. 1.—Twelve small lymph nodes were received, eleven showing the criteria of normal nodes with well conserved architecture and numerous follicles. One node only showed obliteration of the normal architecture and a diffuse proliferation of lymphoblasts.

GENERAL DISCUSSION

One hundred and twenty four cases of lymphoid hyperplasia, where clinically and morphologically the picture was more or less uncertain, were re-examined clinically and the slides revised.

The histological revision resulted in a greater differentiation, inasmuch as $\frac{4}{5}$ of the morphologically uncertain cases were classed as either benign or malignant. In $\frac{9}{10}$ of these cases the clinical development actually confirmed the correctness of the revised diagnoses.

One fifth of the biopsies remained morphologically uncertain.

These figures certainly do not indicate the total precision of the diagnostic service of our laboratory regarding lymph gland material, even as regards the question : a benign or malignant condition—as only the initially doubtful cases have been included in the present study.

The general findings therefore show that a biopsy in clinically doubtful cases of enlarged lymph nodes is a very important and useful diagnostic means in deciding the presence of malignancy or not.

Nevertheless, certain limitations exist. First, the biopsy may not be representative. This is a general limitation of technical nature.

Second, the criteria used are not of decisive validity. Pictures resembling malignant conditions may accompany benign diseases. Infectious mononucleosis may be mentioned as an example. Cases of malignancy may on the other hand be presented for diagnosis in stages so early that the criteria are not sufficiently developed to permit a diagnosis.

Third, the present knowledge may not be sufficiently distributed and appreciated, as shown in the cases of large follicles and high number of mitoses.

If our results are now placed in relation to the situation very often presented to the clinician and the pathologist, namely, a doubtful case of lymphoid malignancy, we are forced to take a standpoint of grave consequences for the patient on a very uncertain ground.

If the clinical picture greatly points in the direction of malignancy (in cases of lymphoid leukaemia a blood examination is very important) and likewise the pathologist is liable to place the case as Grade III or IV, it will be justifiable to accept and treat the patient as one suffering from a malignant lymphoid condition.

If, on the other hand, the clinical symptoms are more vague and the pathologist cannot go further than a Grade II suspicion, it may be justifiable not to break the news of a malignant diagnosis to the relatives of the patient, and to delay therapeutic action.

The main support for this conservative attitude is the fact, that malignant lymphoid conditions are scarcely ever definitely cured. A delay in diagnosis probably does not jeopardize the final chances of the patient.

If, on the other hand, the patient is not actually suffering from a malignant condition the stigma with the ensuing psychological stress or shock, the reduction of vitality and the exposure to serious means of treatment are of such grave consequence that they cannot be outbalanced by the possible loss of an "early diagnosis" and thereby an early treatment.

This is the philosophy behind our teaching and our discussions with the clinicians in cases of the type discussed in the present paper.

In spite of an intensive and often uncritical propaganda, the Norwegian clinicians seem to support this philosophy. Of the 41 patients with a primary diagnosis of hyperplasia with a moderate suspicion of malignancy (Grade II) 9 only were given specific treatment, possibly those with the most marked clinical symptoms. Five of these 9 are alive symptom-free, 4 have developed a malignant lymphoid condition. More important, out of the 32 untreated patients 27 are symptom-free, 2 died from an intercurrent disease and 3 only have manifested a malignant lymphoid condition.

It seems that the total benefit of the conservative attitude has been greater than the total possible loss of an early and intensive treatment.

For the patient a very close contact between clinician and pathologist is of utmost importance.

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TUMOUR CELLS IN THE BLOOD WITH SPECIAL REFERENCE TO PRE- AND POST-HEPATIC BLOOD

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BLOOD stream metastasis of malignant tumours has long been recognised. Over the years there have been sporadic reports of atypical cells in the blood of cancer patients but it is only with the advent of techniques for isolating different types of cells from whole blood by utilising minor differences in their gravity (Fawcett and Valle, 1952) that it has been possible to demonstrate definitely such tumour cells.

Engell (1955) reported that in 59 per cent of patients with carcinoma of the rectum or colon either definite malignant cells, or atypical cells suggestive of malignant cells, were demonstrable in the venous blood draining the tumour area. He also found tumour cells in the blood draining other tumour sites and in the peripheral blood. Other investigators, using slightly different separation techniques, have confirmed Engell's findings. Most noteable of these are Sandberg and Moore (1957) and Sandberg *et al.* (1958), whose technique depends on the rapid sedimentation of erythrocytes with bovine fibrinogen, and Roberts *et al.* (1958) and Cole *et al.* (1958) who refined this technique by layering the tumour cells at an albumin interface of known specific gravity after sedimentation of the erythrocytes with bovine fibrinogen.

Our own work falls into two parts. We first of all investigated patients with a variety of tumours, but particularly with tumours of the parotid and breast. Having found that we were able to identify tumour cells both in the blood draining tumour sites and in the peripheral venous blood, we have attempted to determine to what extent tumour cells are filtered from the blood by the liver in patients either with primary gastro-intestinal carcinoma or with abdominal secondary deposits.

METHOD

For the second part of our studies blood was taken before and after it entered the liver. Fig. 1 demonstrates the way in which the post-hepatic samples were taken. A catheter is passed under fluoroscopy through the right side of the heart and into the right and then the left hepatic vein, and a 5 ml. sample of blood is taken from each vein. At the same time 5 ml. sample of peripheral blood is also taken. As it is difficult to take pre- and post-hepatic blood simultaneously at operation, the post-hepatic is taken pre-operatively. The pre-hepatic samples are taken at operation from the portal vein as soon as the abdomen is opened and with minimal handling of the tumour. Again a simultaneous 5 ml. of peripheral blood sample is taken.

The samples are processed immediately using a slight modification of Sandberg and Moore's (1957) technique. The blood is put directly into a tube containing 1 mg. of heparin and 80 mg. of bovine fibrinogen in 2 ml. of water, and allowed to stand until the erythrocytes sediment. The supernatant fluid is then pipetted off and centrifuged at 1000 r.p.m. for 5 minutes. The supernatant plasma is decanted and the residual portion of cells resuspensed in the remaining fluid. A known aliquot of this suspension is smeared on four slides and stained with Leishman's stain ; two are counter-stained with peroxidase, which provides a

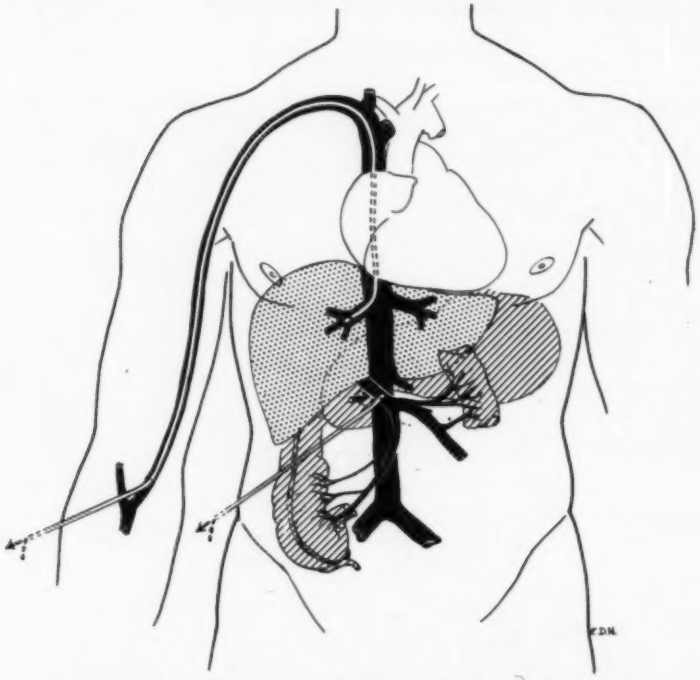


FIG. 1.—Diagram showing catheters in position for taking hepatic and portal samples of blood.

contrasting background in that neutrophils are peroxidase-positive while tumour cells, lymphocytes and monocytes are peroxidase-negative. Examination of approximately one-fourth of one peroxidase, and one fourth of one non-peroxidase stained smear for each sample was then carried out by one of the authors (W.S.F.) and the possible malignant cells marked. These were reassessed by the other author (J.S.) and possible blood cells excluded.

The criteria for determining whether cells are tumour cells have been discussed by numerous authors (Engeli, 1955 ; Sandberg and Moore, 1957 ; Roberts *et al.* 1958). Clumps of large atypical cells with large hyperchromatic nuclei in which one or more nucleoli can be seen contribute the classical picture. In our cases whenever possible a tumour smear was made for comparison.

Smears are classified as definitely positive and negative, but there remain a number which contain atypical hyperchromatic non-blood cells which cannot be definitely identified as malignant. These we have classified as suspicious. Fig. 2–7 show examples of the types of cells we have seen.

Results

Our complete results in all the 51 patients that we have investigated to date are given in Table I. The 4 cases of mixed tumour of the parotid and the one case of benign gastric ulcer served as controls, and as expected showed no tumour cells in the blood. It will be seen that tumour cells or suspicious cells were found in a significant proportion of all cases either in regional vein blood, or in peripheral vein blood, or in both. The frequency varied in the different groups, and was highest in the regional vein blood in cases of tumours of the gastro-intestinal tract, though the figures are too small for final conclusions on this point.

Further Analysis of the Pre- and Post-Hepatic Blood Studies

Fourteen patients had blood taken from the hepatic veins. Five, a heterogeneous group including cases of secondary deposits in the liver from carcinoma of the breast, did not have portal vein blood taken; none of these 5 patients showed tumour cells in the hepatic vein blood. Nine patients had samples taken both of pre-hepatic (i.e. portal) and of post-hepatic (i.e. hepatic vein) blood. In 4 cases the portal blood was positive for malignant cells and in a further 4 suspicious, making a total of 8 out of 9 cases positive or suspicious. The hepatic vein blood was positive in one case and suspicious in a further case, making a total of 2 out of 9 positive or suspicious. One of the 9 patients, an advanced carcinoma of the

TABLE I.—*Results of Examination of Regional, Peripheral and Hepatic Blood Samples for Tumour Cells*

		A			B			C			D		
		Regional vein blood			Peripheral (1) vein blood			Hepatic vein blood			Peripheral (2) vein blood		
	Number of cases	Pos.	Susp.	Pos. + Susp.	Pos.	Susp.	Pos. + Susp.	Pos.	Susp.	Pos. + Susp.	Pos.	Susp.	Pos. + Susp.
Ca. breast	19	3/15	3/15	6/15	3/10	2/10	5/10	0/2	—	—	—	—	—
Ca. parotid	4	2/4	0/4	2/4	—	—	—	—	—	—	—	—	—
Mixed parotid tumour	4	0/4	0/4	0/4	—	—	—	—	—	—	—	—	—
Gastro-intestinal Ca.	17	8/15	4/15	12/15	1/11	2/11	3/11	1/14	1/14	2/14	1/9	2/9	3/9
Miscellaneous Ca. (Thyroid, bone, kidney, lip, melanoma)	6	2/4	1/4	3/4	0/3	1/3	1/3	—	—	—	—	—	—
Benign ulcer	1	0/1	0/1	0/1									

Results expressed as No. Pos. or Susp./No. patients sampled.
Total cases = 51.
Pos. = Positive for tumour cells.
Susp. = Suspicious of tumour cells (hyperchromatic non-blood cells).
Peripheral vein blood (1) = All cases in which peripheral vein samples were taken.
Peripheral vein blood (2) = Cases which had in addition peripheral vein samples at time of portal samples.

stomach with secondary deposits in the liver, showed large numbers of tumour cells in blood taken from all sites—portal vein, hepatic vein and peripheral venous blood, but they were much more numerous in the portal than in the hepatic blood.

DISCUSSION

Our results confirm those of Engell (1955), Moore, Sandberg and Schubarg (1957), and Cole *et al.* (1958) in showing that tumour cells can frequently be demonstrated in the blood in cases of malignant disease. Though our figures are too small and the cases too heterogeneous for precision, both in site of growth, malignancy and degree of spread the entrance of tumour cells into the blood stream in malignant disease is clearly a frequent event, particularly since their demonstration represents the situation in a small sample at the moment of sampling only. Blood borne metastasis, though frequent, is not as frequent as this, and most tumour cells which get into the blood stream must be destroyed. The possibility exists that the attempted destruction of cells in the blood stream by chemotherapeutic agents might make matters worse by lowering the resistance of the normal tissues unless selective agents were discovered.

The pre- and post-hepatic blood investigations show that the liver is a highly, but not completely, efficient filter of tumour cells reaching it by the portal vein, a fact already well known both from morbid anatomy and from experimental studies (Patey, 1937). If the one patient is excluded who had large numbers of malignant cells in all samples and who might be likened to an *in vivo* tissue culture of malignant cells, 7 out of 8 portal vein blood samples contained either tumour cells or suspicious cells, while only one of 8 samples of hepatic vein blood from the same patients was suspicious of tumour cells.

Though again the figures are too small to be significant, it will be noted that the peripheral vein blood contained tumour cells or suspicious cells more frequently than the hepatic vein blood, both in the samples taken before operation (Table I, Column B) and in those taken at operation (Table I, Column D). If this finding were confirmed, it would suggest either that tumour cells are released from the liver sporadically and that the finding of them in the hepatic vein depended on the chances of timing; or that the cells enter the systemic blood stream by another route. An obvious alternative route would be through lymphatics and the thoracic duct.

EXPLANATION OF PLATE.

FIG. 2.—Seven tumour cells from a patient with carcinoma of the stomach. Also several peroxidase-positive neutrophils as well as erythrocytes and lymphocytes.

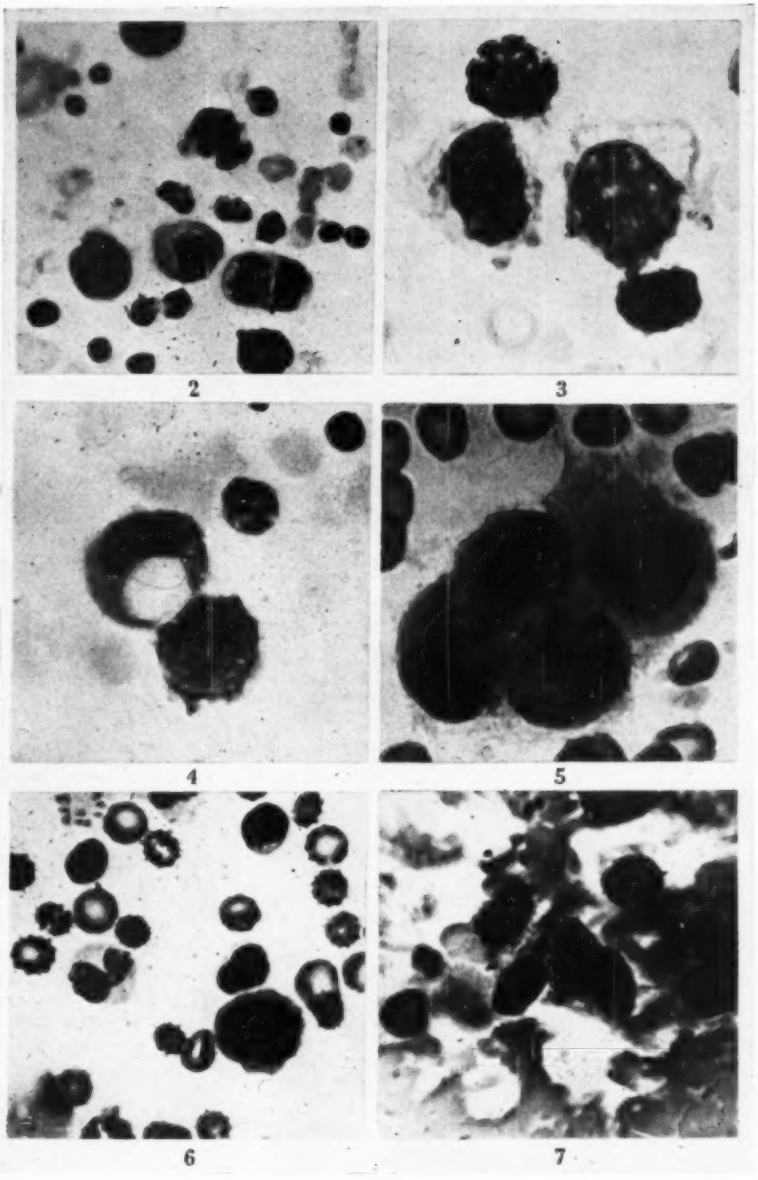
FIG. 3.—Two malignant cells from the same patient with a peroxidase-positive neutrophil near by for comparison.

FIG. 4.—Three tumour cells, one of which is a typical signet ring cell from the same patient as Fig. 2 and 3.

FIG. 5.—A large clump of tumour cells from the external jugular vein of a patient with carcinoma of the parotid.

FIG. 6.—An atypical hyperchromatic non-blood cell.

FIG. 7.—A rolled up sheet of endothelial cells scraped from a vein at autopsy. Note pale staining, and, although poorly seen in reproduction, the very pale cytoplasm holding the cells together.



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SUMMARY

1. Tumour cells or suspicious cells were found in a significant proportion of 51 cases, both in blood draining malignant tumour sites and in peripheral venous blood.

2. Tumour cells or suspicious cells were found in the portal blood in 8 out of 9 cases of carcinoma of the gastro-intestinal tract, but in only 2 of the same cases in hepatic vein blood.

3. There is a suggestion that some tumour cells from carcinomas of the gastro-intestinal tract may reach the systemic circulation by the lymphatic route.

The authors wish to express their sincere thanks to Mr. D. H. Patey for the concept of pre- and post-hepatic studies, permission to study the above patients and for his very helpful suggestions regarding the text of the article. In addition we wish to thank the surgeons of the Department of Surgical Studies for the taking of blood samples and Dr. J. N. Pattinson for radiological guidance in the performance of hepatic catheterization. One of us (W.S.F.) is in receipt of a grant from the American Cancer Society, and part of the expense of this investigation was defrayed by the British Empire Cancer Campaign.

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RETICULIN PATTERNS IN TUMOURS OF LYMPHOID TISSUE

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In a previous communication (Mackenzie 1958) the diagnostic value of reticulin patterns in carcinomas and sarcomas was discussed. Lesions falling under the general heading of tumours of lymphoid tissue were not included. The purpose of the present article is to inquire into the diagnostic value, if any, of a study of the reticulin content and patterns in these lesions. As before, reticulin refers to the argyrophilic fibres present within the neoplasm.

Any inquiry into diseases of lymphoid tissue encounters the major difficulty of terminology. Ignorance of aetiology and profound differences of opinion regarding the neoplastic nature of certain lesions have resulted in a complex nomenclature. Not only do the well-recognised conditions have a variety of names but certain rarer ones, such as reticular lymphoma, are not considered as definite separate entities by some authorities. In addition, the differentiation of such a lesion from Hodgkin's disease depends on a number of distinctions so fine that, in some cases, no unanimity of opinion would be obtained on a given section amongst a group of observers—however experienced. Thus, in discussing reticulin patterns, it is only possible to describe the distribution of argyrophilic fibres in typical examples of conditions generally accepted as entities. The terminology used here is that of Lumb (1954). It has the advantage of being simple and, in the present state of our knowledge, loses nothing by that simplicity.

The normal lymph-node

In order to be able to assess abnormal reticulin patterns in lymph-nodes, a knowledge of the normal range of variability is essential. As Marshall (1956) has rightly pointed out, the so-called " normal " lymph-node is exposed to a wide variety of stimuli and consequently shows a wide degree of morphological variation. Thus, any description of a normal node refers to some extent to an abstract and idealised structure. In addition to text books on histology, the anatomy of lymph-nodes has been described by Robb-Smith (1938) and Marshall (1956). Robb-Smith pointed out that, within a normal gland, only three structures differed histologically: (1) the sinuses; (2) the follicles; and (3) the reticular tissue, wherever it lay in the gland. Reticulin fibres penetrate all parts. They form a heavy network on the inner surface of the capsule, on the trabeculae which pass inwards from the capsule and around the adventitia of the arteries and veins. They are fairly plentiful throughout the reticular tissue but are less prominent in the sinuses. Reticulin fibrils are seen within both true and pseudo follicles though, as a rule, they are scantier in the former type. An anatomical diagram of a normal lymph-node is shown in Fig. 1.

Through the years, a number of writers have commented upon reticulin patterns in lesions of lymphoid tissue and references are given in each section. In the present

series 305 cases were studied and these are considered under the headings shown in Table I. The sections were stained by haematoxylin and eosin and by a slightly modified version of Gomori's silver impregnation technique.

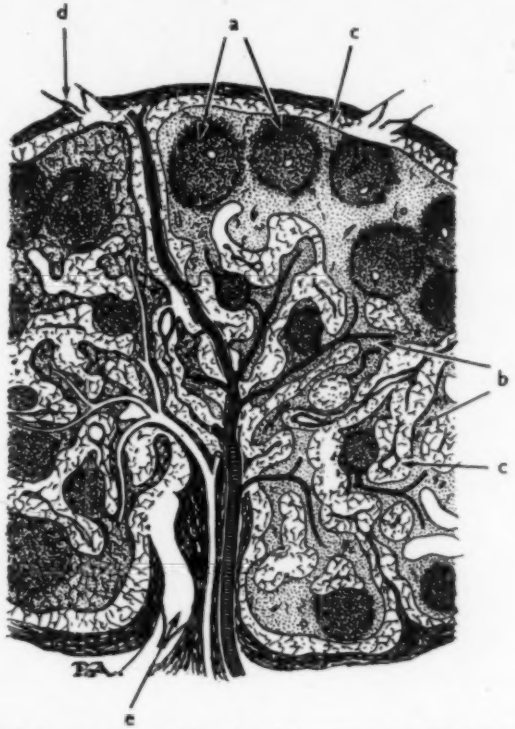


FIG. 1.—Anatomical diagram of normal lymph node (modified after Heudorfer (1921) *Z. Anat. u. Entwickl.* **61**, 365).

(*a*) Primary follicles. (*b*) Medullary pulp. (*c*) Sinuses. (*d*) Afferent lymphatic. (*e*) Efferent lymphatic.
The reticulin framework of the node is shown on the left side of the diagram.

TABLE I

Type of tumour	Number of cases
Hodgkin's disease	147
Lymphosarcoma	65
Anaplastic sarcoma of lymphoid tissue	35
Reticulum cell sarcoma	27
Follicular lymphoma	21
Reticular lymphoma	10
Total	305

Hodgkin's disease (lymphadenoma, Hodgkin's granuloma, fibromyeloid medullary reticulosis)

Hodgkin's disease is the commonest of the tumours of lymphoid tissue and the reticulin content has been described in many communications. Pullinger (1932), Jackson and Parker (1947), Robb-Smith (1947), Lumb (1954) and Marshall (1956) have all commented on the reticulin increase which is nearly always present. Robb-Smith (1938) noted that, occasionally, there is collagenous increase without a corresponding increase in reticulin. Harrison (1953) noted scanty reticulin in certain rapidly growing lesions, while Lumb (1954) states that an increase in the argyrophil network precedes the laying down of collagen.

The present series contained 147 cases. Eighty showed a gross increase in the reticulin content of the gland (Fig. 2). In 62 cases the reticulin was unmistakably increased, though to a lesser degree. Thus 96 per cent showed some reticulin increase. In only five cases of undoubted Hodgkin's disease was the reticulin content approximately within normal limits as regards quantity. In all cases studied the distribution was abnormal and the gland architecture was destroyed. There was no precise correlation between the cellularity of the disease and the reticulin content, although the reticulin tended to be less in the rapidly growing lesions. Occasionally glands showed dense collagen formation while the remaining cellular areas contained only a few reticulin fibrils (Fig. 3).

Lymphosarcoma (lymphocytoma and lymphoblastoma)

This term is widely accepted to describe a lymphoid tumour made up of lymphocytes or lymphoblasts or a mixture of the two. Comments on the reticulin content have been made by Robb-Smith (1938), Jackson and Parker (1947), Dukes and Bussey (1947), Harrison (1953), Lumb (1954) and Evans (1956). There has been universal agreement that the reticulin is seldom increased and often shows a decrease.

There were 65 cases in the present series. In 33 cases there was a considerable reduction in the total reticulin content while in 27 cases the reduction was less apparent. Thus 92 per cent showed some decrease. The most typical picture showed destruction of gland architecture with reticulin fibrils of varying size scattered, often at wide intervals, between the tumour cells (Fig. 4). Lymphosarcomas developing from follicular lymphomas often showed a remnant of follicular pattern. In 5 untreated cases of lymphosarcoma the reticulin was increased (Fig. 5).

EXPLANATION OF PLATES

FIG. 2.—Hodgkin's disease. Greatly increased reticulin. Silver impregnation × 95.

FIG. 3.—Hodgkin's disease (sclerotic type). Silver impregnation × 25.

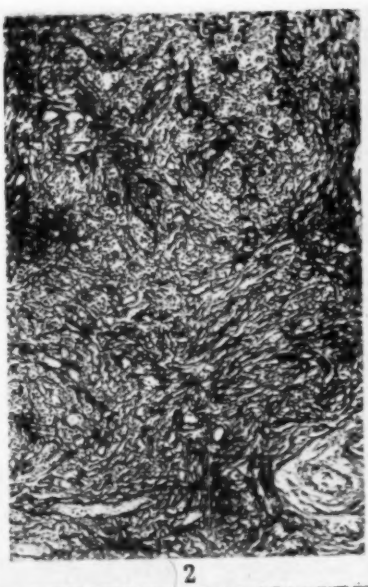
FIG. 4.—Lymphosarcoma. Diminished reticulin. Silver impregnation ×165.

FIG. 5.—Lymphosarcoma. This amount of reticulin might well be seen in a reticulum cell sarcoma. Silver impregnation × 110.

FIG. 6.—Reticulum cell sarcoma. Reticulin not greatly increased. Silver impregnation × 110.

FIG. 7.—Reticulum cell sarcoma. Greatly increased reticulin. Silver impregnation × 165.

FIG. 8.—Follicular lymphoma. Condensation of reticulin around follicles. Silver impregnation × 55.



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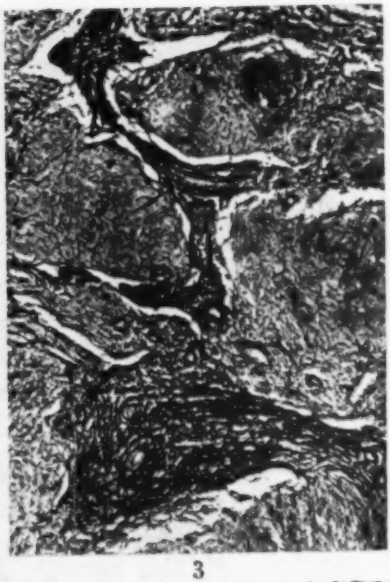

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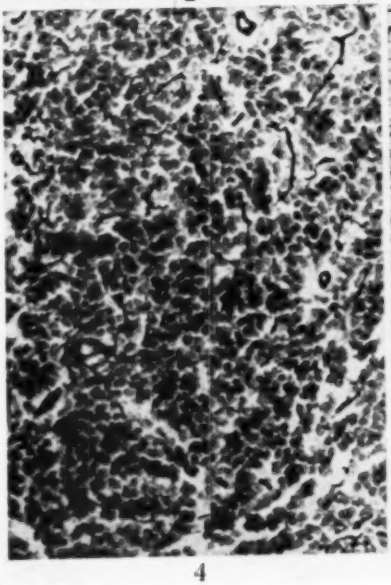

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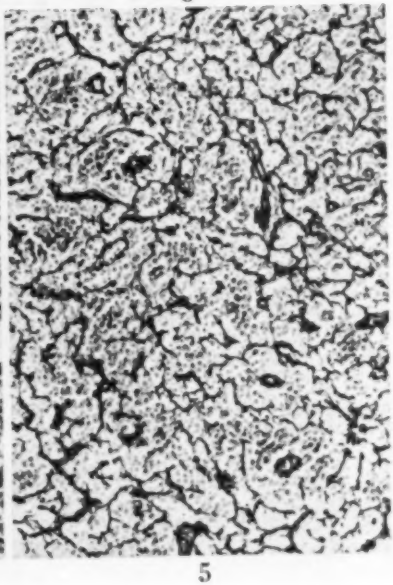

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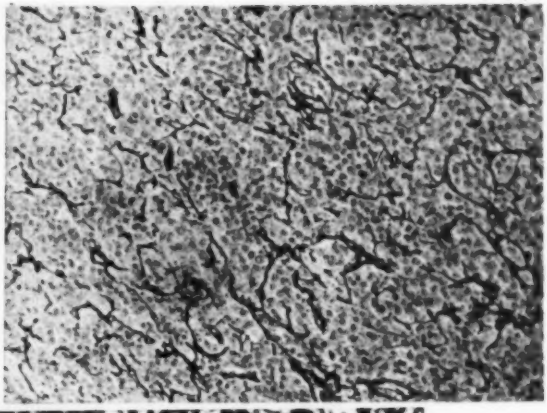

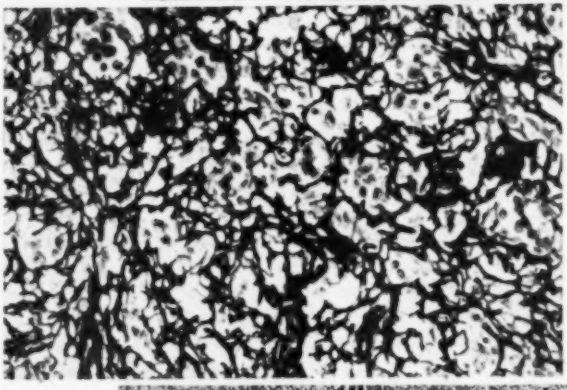

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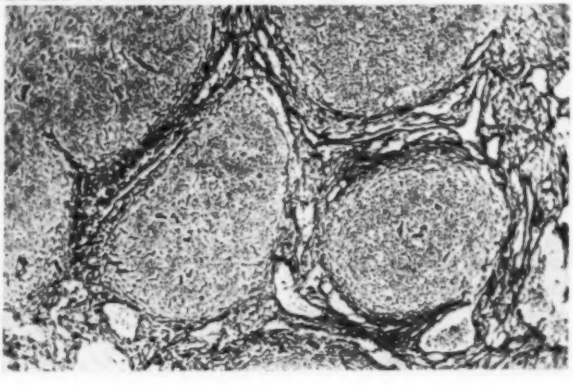

Anaplastic sarcoma of lymphoid tissue (Hodgkin's sarcoma, lymphoblastic reticulosarcoma, stem cell sarcoma and others)

This group includes the anaplastic variants of the other types. The reasons for collecting these anaplastic and pleomorphic tumours into a simple group have been given by Lumb (1954). The principal cells involved are immature forms of the lymphocyte and reticulum cell and giant forms, multi-nucleated cells and mitotic figures are often seen in large numbers. Under various names these tumours and their reticulin contents have been discussed by Callender (1934), Robb-Smith (1938), Jackson and Parker (1947), Harrison (1953), Evans (1956) and others.

There were 35 cases in the present series. With such a wide variation in the cytology it was not surprising to find an equally wide variation in the reticulin pattern and content. In addition the reticulin density often varied considerably in different parts of a gland. Areas showing only a few scattered fibrils lay next to other fields where there was a dense network of reticulin. Sclerosis and collagen formation were also seen. The variability of reticulin pattern within a gland often made an assessment of the total content difficult. However in 21 cases there appeared to be a considerable overall increase in reticulin while 11 showed a more moderate one. In two very cellular tumours the reticulin was decreased.

Reticulum cell sarcoma [reticulo-sarcoma, lymphosarcoma (reticulum cell type) and others]

The difficulty of terminology is particularly apparent in any discussion of reticulum cell sarcoma. In this context the term is used in the manner of Warren and Picena (1941) and Lumb (1954). It refers to a tumour made up of malignant reticulum cells which, though they may show some pleomorphism, are yet sufficiently uniform in type for the lesion to be distinguished from the anaplastic sarcomas. Harrison (1953) described a similar lesion under the title: reticulosarcoma. Other writers who have described the reticulin patterns of lesions made up predominantly of reticulum cells include Callender (1943), Robb-Smith (1938) and Jackson and Parker (1947).

Twenty-seven cases were studied. In 16 cases there was a marked increase in reticulin. Eight cases showed a more moderate increase while in 3 glands the reticulin content appeared approximately within normal limits. Thus 89 per cent showed some reticulin increase though the range was wide (Fig. 6, 7). In all cases except one, silver impregnation showed complete destruction of the gland architecture. In the one case only part of an enlarged gland was involved and the contrasting reticulin patterns of the normal and neoplastic areas were clearly seen.

The reticulin patterns were of the sarcomatous fibrillary type and the reticulin density was often constant throughout a gland. The fibrils were usually fine and scattered diffusely amongst the cells. Sometimes individual prolongations joined one cell with another and occasionally single cells were surrounded by a sheath of reticulin. Sclerosis and collagen formation were not observed.

Follicular lymphoma (giant follicular lymphoblastoma, lymphoid follicular reticulosis and Brill-Symmer disease)

This is another lesion to which several names have been given. The first detailed accounts were those of Brill, Baehr and Rosenthal (1925) and Symmers

(1938). Other communications on the subject have been those of Symmers (1942), Baggenstross and Heck (1940), Gall and Mallory (1942), Robb-Smith (1938 and 1947), Wetherby-Muir, Smith and Anderson (1952), Harrison (1953) and Lumb (1954). These writers noted that silver impregnation clearly emphasised the follicular pattern and that there was often a condensation of reticulin round the periphery of the follicles. Within the follicles the reticulin was stated to be scanty or absent. Rappaport, Winter and Hicks (1956) have subdivided follicular lymphoma into five types and noted variations in the reticulin patterns.

There were 21 cases in the present series. The pattern was quite different from that of the other lesions described. Follicular structures were present throughout the substance of the glands and there was often severe compression of the sinuses and medullary tissue. Silver impregnation clearly demonstrated these structures which were not always clearly apparent in sections stained with haematoxylin and eosin. Within the follicles themselves there were often a number of tiny fibrils while a dense condensation of reticulin round the periphery was a common finding and was seen in 13 cases (Fig. 8). The writer is in agreement with Rappaport that this condensation is seen when the follicles are closely packed and may also occur in reactionary hyperplasia. When sarcomatous change supervenes in follicular lymphoma a break occurs in the ring of peripheral reticulin and the follicles become increasingly ill-defined. Eventually, even with silver impregnation, they are no longer discernible and the reticulin pattern becomes that of the more malignant form, often a lymphosarcoma.

Reticular lymphoma (*Hodgkin's paragranuloma and lymphoreticular medullary reticulosis*)

This is the rarest of the lymphoid tumours and its existence as an entity entirely separate from Hodgkin's disease is doubted by some authorities (Marshall, 1956). The reticulin content which most workers have found to be within normal limits or slightly increased has been described by Jackson (1937), Jackson and Parker (1944*a*, *b*, 1947), Robb-Smith (1947), Harrison (1952) and Marshall (1956). Harrison drew particular attention to the lobulation which was present in all his cases. The glands were divided into large lobules by collagen and subdivided into smaller lobules by reticulin.

Ten cases were available for study. The gland architecture was destroyed in all cases and in five of them the lobulation described by Harrison was observed. In these cases the intra-lobular reticulin was relatively scanty and made up of scattered short fibrils of varying thickness. In the other five cases scattered fibrils were seen throughout the glands. In amount the reticulin was considerably less than that observed in the vast majority of cases of Hodgkin's disease and somewhat greater than in typical lymphosarcoma.

DISCUSSION

The reticulin in tumours of lymphoid tissue can be studied from the points of view of distribution and amount. Harrison (1958, personal communication) stresses the great importance of using a silver impregnation method which clearly defines the argyrophilic fibres and does not allow nuclear staining to mask the smaller details. He also draws attention to the information which can be gained

by use of a very low power, such as a × 10, hand lens in the initial examination of a section.

The distribution of reticulin

A study of the reticulin pattern is of more value than any assessment of the amount present. A low power view will show whether the basic structure of the gland architecture is preserved or not. The number, size and distribution of any follicles present can be clearly seen and their relationships to the sinuses determined. Although wide variations in pattern frequently occur, certain distributions are characteristic. In Hodgkin's disease and in the anaplastic sarcomas the reticulin density is usually uneven, and dense masses of reticulin may lie adjacent to areas containing only a few fibrils. To a lesser extent this is true of reticular lymphoma. Lymphosarcomas and reticulum cell sarcomas often show a fairly uniform pattern. In follicular lymphoma the reticulin distribution is quite unlike any other lymphoid tumour. Unfortunately the pattern may closely resemble that seen in gross reactionary hyperplasia and is of little use in distinguishing these two conditions. Apart from the primary lymphoid tumours, silver impregnation will often reveal the site of abnormal cells. For example, Robb-Smith (1947) points out that, occasionally, in children with sinus catarrh of lymph-nodes, the histiocytes become multinucleated and this giant cell sinus reticulosis may well suggest a malignant process. Silver impregnation reveals that these multinucleated cells are limited to the sinuses and follow up studies have suggested that this is a benign condition. Similarly, a metastasis can often be distinguished from a primary lymphoid neoplasm. With early invasion the tumour cells are confined to the sinuses while, in later cases, the invading tumour will possess its own reticulin pattern and will often be identifiable as a carcinoma.

The amount of reticulin

Marked increases or decreases in the amount of reticulin in a lesion are easily observed. Great caution is necessary before any importance is attached to minor changes. The reticulin content of a tumour is often variable from one part to another and the overlap between a number of tumours in this group is considerable. For example a reticulum cell sarcoma with a minimal reticulin content for this type of lesion, a reticular lymphoma or a lymphosarcoma with more than average reticulin may be virtually indistinguishable by silver impregnation methods. Thus, while an increase of reticulin may help to confirm a diagnosis of reticulum cell sarcoma, a decrease does not exclude it. It must also be remembered that the predominating cell type and the general histological picture may change as the disease progresses. This has been discussed by Custer and Bernhard (1948) and other writers. Such a change may be accompanied by a change in the reticulin content. The most that a study of the gross amount of reticulin can do is to provide confirmatory evidence for a given diagnosis or, occasionally, to suggest an alternative one.

SUMMARY

1. The reticulum content and pattern in 305 cases of tumours of lymphoid tissue have been examined.

2. The definite but limited value of silver impregnation in the diagnosis of these tumours has been discussed.

I wish to thank Sir Stanford Cade for permission to study many cases under his care. I am indebted to Lt.-Col. P. D. Stewart, R.A.M.C., and W/Cdr. R. M. Cross, R.A.F., for the loan of blocks ; to Springer-Verlag for permission to reproduce Fig. 1 and to the Department of Medical Photography, Westminster Hospital.

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HORMONE EXCRETION IN PROSTATIC CANCER: AN ATTEMPT TO CORRELATE URINARY HORMONE EXCRETION AND CLINICAL STATE

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In a previous paper the excretion of oestrogens, 17-oxosteroids and 17-oxogenic steroids by men with untreated prostatic cancer was compared with that of controls of the same age, and the effects of treatment by synthetic oestrogens or by castration were briefly described (Bulbrook, Franks and Greenwood, 1959). The present paper describes in detail the changes which occur within the first two years of treatment of prostatic cancer by castration or by the administration of synthetic oestrogens and attempts to correlate the clinical effects of such treatment with the changes in hormone excretion in eight patients.

MATERIAL

Eight patients with prostatic cancer, (aged 66 to 80 years), were admitted to hospital and 24 hour urine specimens collected for three to fifteen days before treatment. The patients were re-admitted to hospital at intervals during treatment for clinical evaluation and urine collections. The clinical diagnosis was confirmed histologically in five cases and cytologically (prostatic smear) in the remaining four. Treatment was by surgical castration (three patients) or by synthetic oestrogens (five patients). The clinical assessment was made without knowledge of the biochemical findings.

CHEMICAL METHODS

Chemical methods used are as previously described (Bulbrook, Franks and Greenwood, 1959). The effects of synthetic oestrogen treatment on the urinary oestrogen excretion are given for oestrone and oestriol only since the oestradiol-17β fraction cannot be measured accurately in urine from these patients. Intravenous diethyl stilboestrol phosphate also interferes with the measurement of oestrone and in one case where this compound was given only oestriol values are reported.

RESULTS

A brief description of the clinical and biochemical findings is given for each patient. The hormone excretion of the 8 patients is shown graphically in Fig. 1 to 8. Periods of clinical tumour activity are shown by a black bar; periods of remission by a white bar.

All periods of time have been taken from the day of operation or the start of treatment. The mean hormone excretion in the pre-treatment period—the control level—has been used as the baseline for comparing subsequent hormone determinations during treatment. Age refers to that at operation or at the start of treatment.

a. *Castration : its effects on the clinical state and on the excretion of oestrone, oestradiol-17β, oestriol, 17-oxosteroids and 17-oxogenic steroids. (Group of three patients)*

Patient No. 1, *aged* 66 *years*, was admitted with incontinence and supra-pubic pain. The prostate was clinically malignant and a prostatic smear contained malignant cells. The serum acid phosphatase (formalin inactivated) was 3·0 units. There were no skeletal metastases on X-ray examination. After a control period of 9 days the patient was treated by castration and per-urethral resection. The resected tissue contained areas of anaplastic carcinoma. The changes in hormone excretion are shown in Fig. 1. In the immediate post-operative period there was a marked rise in excretion of all three groups of steroids measured. This phenomenon has been frequently observed to follow operation and is probably due to an adrenal cortical reaction to stress. After this initial rise the amounts of excreted oestrogens and 17-oxosteroids fell progressively to pre-operative levels. Oestrogens were not detected in the urine 14 weeks after operation, and the 17-oxosteroid excretion was very low. Clinically there was a marked improvement with complete freedom from symptoms. Over the period from 33 to 65 weeks after operation, oestrogen was again detectable in the urine increasing to pre-operative levels and accompanied by an increase in 17-oxosteroid secretion. This period was characterised by the appearance and subsequent development of metastases in the pelvis and spine. When the patient complained of pain, 72 weeks after castration, stilboestrol was given. This was followed by rapid objective and subjective improvement and a second fall in oestrogen and 17-oxosteroid levels. The excretion of 17-oxogenic steroids remained unchanged throughout the period of observation.

Patient No. 2, *aged* 70 *years*, was admitted complaining of urinary difficulty for 6 months. Clinically the prostate was malignant and prostatic smears contained malignant cells (confirmed by later biopsy). The serum acid phosphatase was 1·5 units. There were no skeletal metastases on X-ray examination. The patient was castrated and the post-operative hormonal changes are shown in Fig. 2.

A transient rise in oestrogen excretion in the immediate post-operative period was followed by a fall to below the pre-operative levels. Later specimens obtained during follow-up showed a return of excretion to pre-operative values. The 17-oxosteroid levels showed no significant change at operation or in the following 46 weeks, whereas the 17-oxogenic steroids showed a slow fall after operation to low levels but with one exceptionally high value. Clinically the operation resulted in the disappearance of urinary symptoms and an arrest in the tumour growth up to 44 weeks. The re-appearance of mild urinary symptoms was noted some 32 weeks after the return of oestrogen excretion to pre-operative levels, and on examination the prostate was enlarged and hard. This patient died 78 weeks after castration with left ventricular failure. Histologically the prostate removed at autopsy showed many areas of growing tumour.

Patient No. 3, *aged* 70 *years*, had a retropubic prostatectomy when 63 years old. Histologically there was an area of prostatic carcinoma in the tissue removed. No treatment was given.

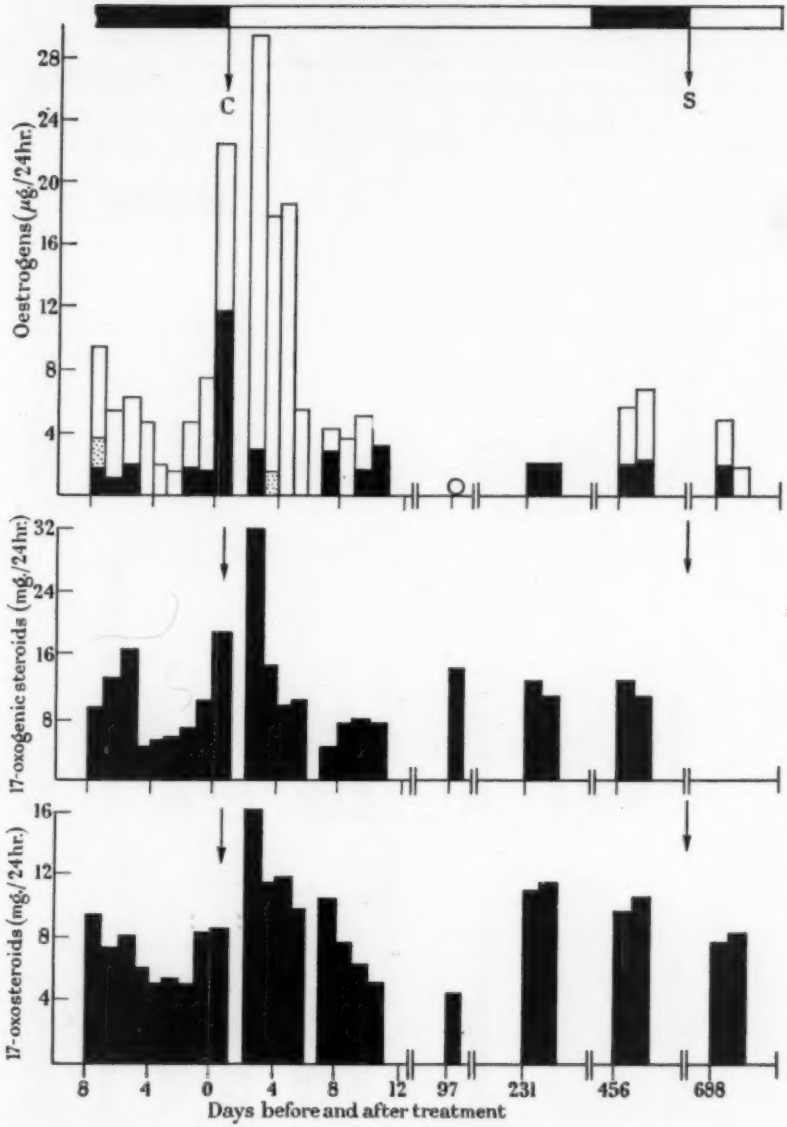

FIG. 1.—The effect of castration on the excretion of oestrogens, 17-oxosteroids and 17-oxogenic steroids.

Patient 1, aged 66 years.

The vertical arrows show the day of treatment.

C = castration.
S = stilboestrol.

Each vertical column gives the result of a duplicate determination on a 24 hour urine specimen. For the oestrogens, the height of the block shows the total amount excreted, subdivided into oestrone (■), oestradiol (▦) and oestriol (□).

The horizontal bar at the top of the figure indicates clinical state. The black areas represent tumour growth; the white areas represent regression. All subsequent figures are plotted in the same way.

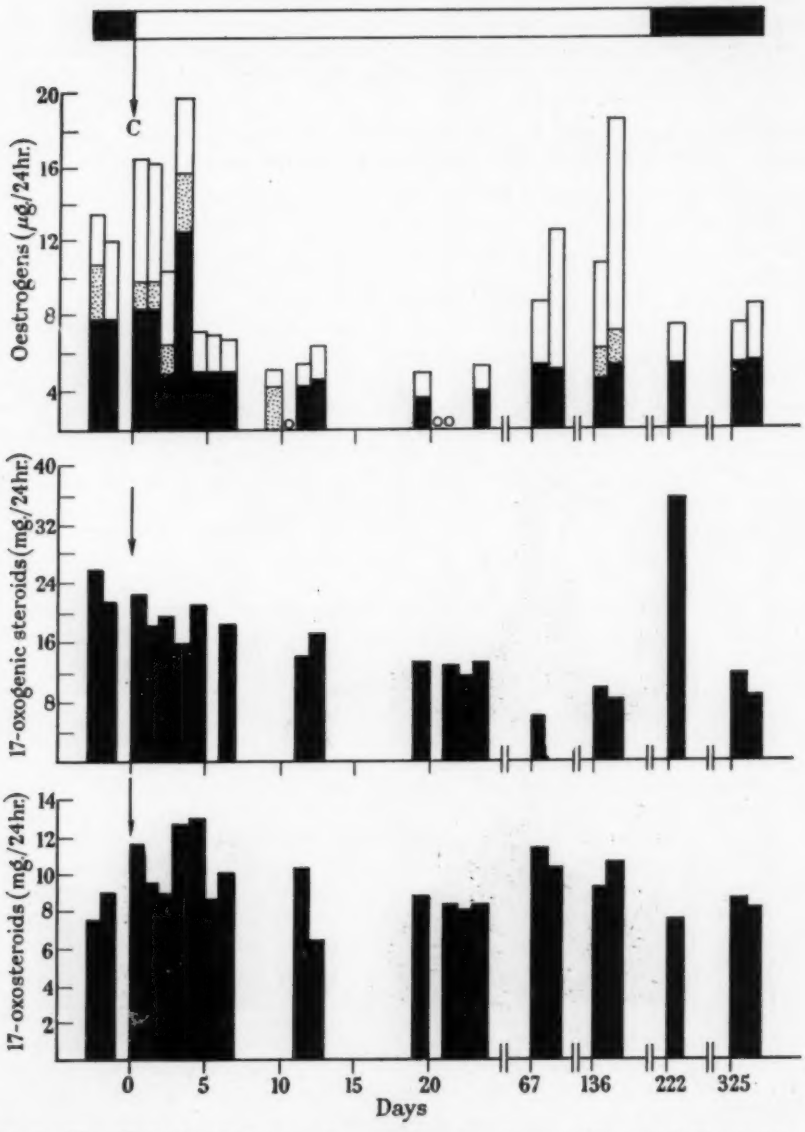

FIG. 2.—The effect of castration on the excretion of oestrogens, 17-oxo-and 17-oxogenic steroids.

Patient 2, aged 70 years.

He was admitted to hospital 7 years later with a two months' history of pain in the right thigh. X-rays showed osteosclerotic deposits in the pelvis, femur and lumbar spine. The serum acid phosphatase was raised (45·8 units). Castration resulted in clinical improvement, a fall in serum acid phosphatase to 2·3 units but no immediate reduction in urinary oestrogens, 17-oxosteroids or 17-oxogenic

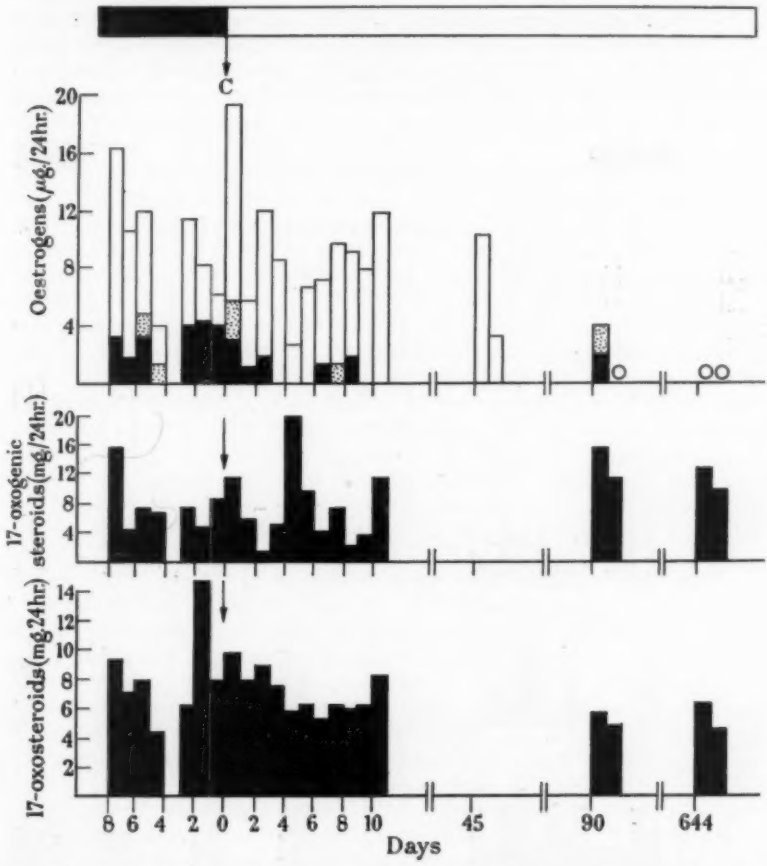

FIG. 3.—The effect of castration on the excretion of oestrogens, 17-oxo- and 17-oxogenic steroids. Patient 3, aged 70 years.

steroids. Clinical improvement and the lowered serum acid phosphatase was maintained up to 92 weeks after operation. During this time oestrogen excretion fell progressively to below pre-operative levels and at the last follow-up no urinary oestrogen was found, whilst the 17-oxosteroids and 17-oxogenic steroids have remained unchanged.

b. *Synthetic oestrogen: the effects of administration on clinical state and on the excretion of oestrone, oestriol, 17-oxosteroids and 17-oxogenic steroids. (Group of five patients.)*

Patient No. 4, aged 69 years, when first seen complained of dysuria and backache, pain on micturition, suprapubic pain and left sciatic pain. The prostate was moderately enlarged, fixed, hard and irregular. A per-urethral resection was carried out. Histological examination showed an adenocarcinoma with moderate fibroblastic reaction. After a 6-day control period, stilboestrol was given (50 mg. twice daily). There was little effect on oestrogen excretion for the first 5 days but thereafter the excretion of oestriol fell progressively (Fig. 4). At the first follow-up

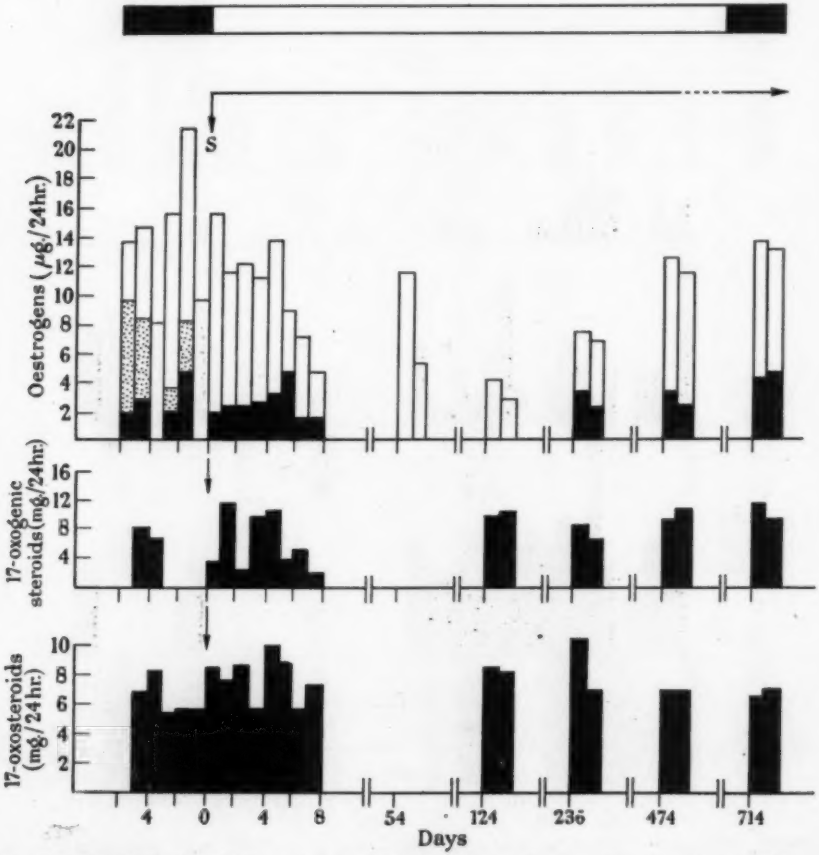

FIG. 4.—The effect of synthetic oestrogen administration on the excretion of oestrogens, 17-oxo- and 17-oxogenic steroids.

Patient 4, aged 69 years.

The dotted break in the line showing the period of stilboestrol treatment denotes a period when the patient stopped taking the drug.

at 8 weeks, the amount of oestriol in the urine almost equalled the control level but after 18 weeks of treatment oestrogen levels were considerably depressed (30 per cent of control mean level). However, during three subsequent follow-up periods oestrogen excretion increased, and at 68 and 102 weeks the amounts were equivalent to the control levels. For some of this time the patient had not taken stilboestrol regularly because of indigestion and subsequently dienoestrol was given (10 mg. three times a day). The fall in endogenous oestrogen excretion after treatment was paralleled by clinical improvement; the rise to control levels was associated with a return of urinary symptoms.

17-Oxosteroid and oxogenic steroid excretion was not altered by treatment. The serum acid phosphatase before treatment was 2·5 units; at the last follow-up, at 102 weeks, it was 1·1 units.

Patient No. 5, aged 78 years, complained of severe frequency of micturition and dysuria. The prostate was small and hard and a prostatic smear contained malignant cells. There were no metastases radiologically. After an 8-day control period, stilboestrol (50 mg. twice daily) was given. During the first few days of treatment both oestrogen and oxosteroid excretion rose but thereafter the amount of oestrogen excreted fell to a very low level, while 17-oxosteroids decreased slightly.

After 8 weeks the stilboestrol was replaced by TACE (chlorotrianisene, 12 mg. t.d.s.). At the first follow-up, 13 weeks after the start of treatment, urinary oestrogens were still barely detectable and by this time 17-oxosteroid excretion was also greatly depressed. At 37 weeks, oestrogen excretion had risen to control levels and 17-oxosteroids had increased slightly. At 73 weeks, the amounts of 17-oxosteroids were approaching the mean control level. The patient's clinical state improved on treatment and the improvement was maintained in spite of the rise in oestrogens and 17-ketosteroids. Serum acid phosphatase levels fell from 7·5 before treatment to 0·4 units by the last follow-up. 17-Oxogenic steroid levels were depressed, and remained so, at all follow-up periods.

Patient No. 6, aged 72 years, had suffered from backache and pain radiating down the left leg for 6 months. The prostate was enormous and craggy, pressing on, but not ulcerating, the rectum. The prostatic smear was positive. The serum acid phosphatase was 120 units; the serum alkaline phosphatase, 92 units. X-ray examination showed metastases in the lumbar and dorsal spine, pelvis, thoracic cage, and the upper end of the left femur. After a 7-day control period the patient was treated by intravenous diethylstilboestrol phosphate for a period of 13 days without benefit. The serum acid phosphatase at the end of this treatment was 122 units; the serum alkaline phosphatase was 82 units. Administration of diethylstilboestrol phosphate made it impossible to measure oestrone and oestradiol-17β in the urine. Oestriol levels were depressed by treatment. 17-Oxosteroid excretion did not change but the level of 17-oxogenic steroids fell to approximately 30 per cent of control values. Diethylstilboestrol phosphate was stopped and stilboestrol (50 mg. b.d.) started. Oestriol excretion declined further and 17-oxosteroid excretion, unaffected by the previous treatment, progressively fell; the 17-oxogenic steroids remained depressed. The fall in hormone excretion was accompanied by an objective regression with decrease of pain. The serum acid phosphatase fell to 75 units and the serum alkaline phosphatase fell to 43 units. Over the following 20 weeks the serum acid phosphatase fell progressively to 15 units, the serum alkaline phosphatase to 18 units. X-ray examination at 18 weeks showed that the metastases were more sclerotic.

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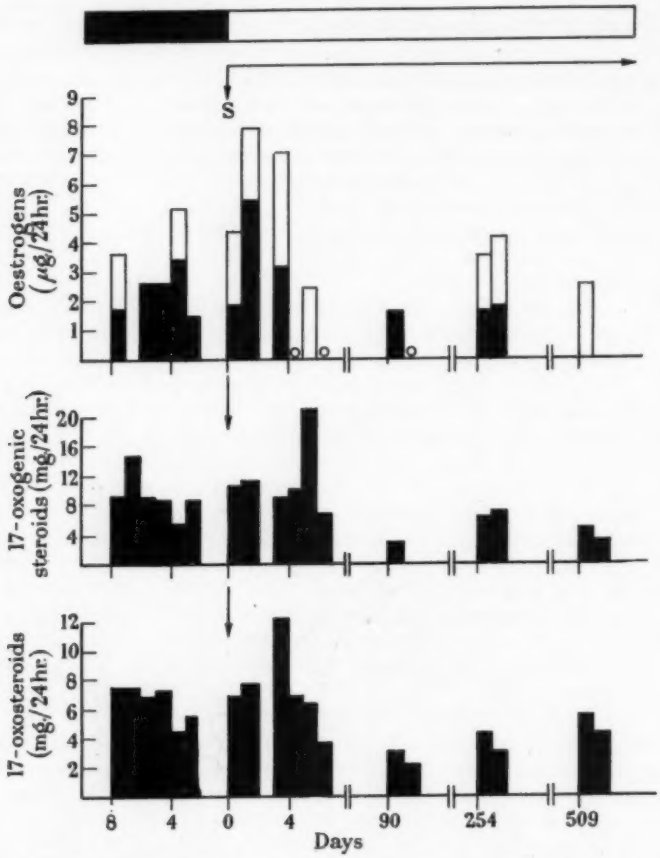

FIG. 5.—The effect of synthetic oestrogen administration on the excretion of oestrogens, 17-oxo- and 17-oxogenic steroids.

Patient 5, aged 78 years.

At the first follow-up (21 weeks) oestrogen levels were still low but 17-oxosteroids and 17-oxogenic steroids were at control levels. X-ray examination showed metastases in the right shoulder. The serum acid phosphatase was now 7·0 units and the serum alkaline phosphatase 12 units.

At the last follow-up (54 weeks), oestrogen excretion had increased and, with the 17-oxosteroids, was at control levels, but 17-oxogenic steroids were depressed. Although the general condition was good the secondary deposits previously noted had developed. The serum acid phosphatase was 3·2 units and the serum alkaline phosphatase was 8·4 units.

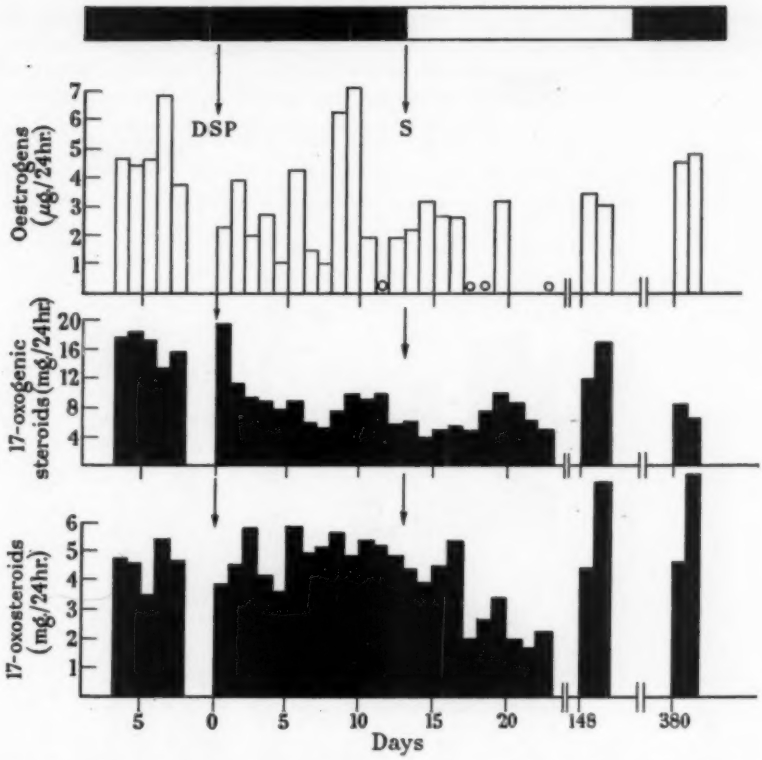

FIG. 6.—The effect of synthetic oestrogen administration on the excretion of oestrogens, 17-oxo- and 17-oxogenic steroids.

Patient 6, aged 72 years.

S = stilboestrol.
D.S.P. = diethylstilboestrol phosphate.

Patient No. 7, aged 80 years, was first admitted with acute retention of urine. A per-urethral biopsy showed one small nodule of prostatic carcinoma of low grade. Serum acid phosphatase was 1·1 units. Fifteen months later there was haematuria and the prostate (per rectum) was hard and clinically malignant. The urine deposit contained malignant cells. TACE (12 mg. t.d.s.) was administered and over the first 10 days of treatment no change occurred in hormone excretion. At the first follow-up (11 weeks) the patient was well. At this stage, oestrogen excretion had fallen to zero; 17-oxosteroid and 17-oxogenic steroid levels were depressed. Serum acid phosphatase was 1·5 units.

At the second follow-up (34 weeks), oestrogen was again detected in the urine but at a level well below that of the control period. 17-Oxosteroids and 17-oxogenic steroids were lower than at the previous follow-up. The serum acid

phosphatase was 0·8 units. The patient remained well but subsequently died of coronary thrombosis.

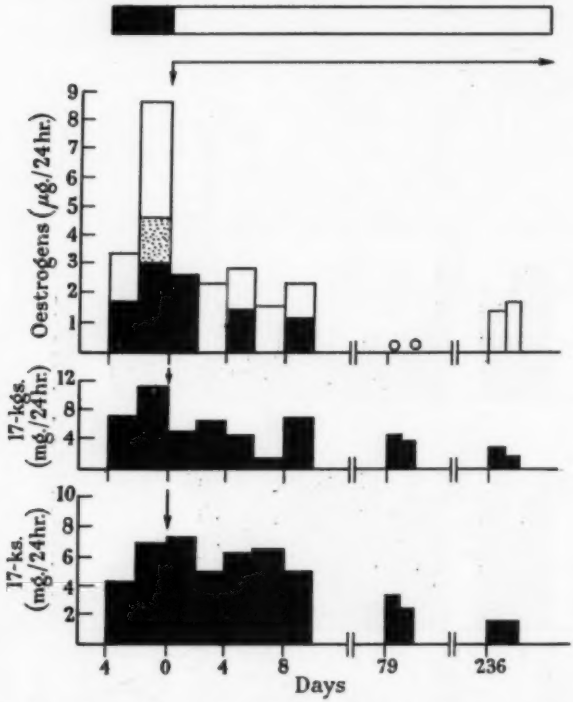

FIG. 7.—The effect of synthetic oestrogen administration on the excretion of oestrogens, 17-oxo- and 17-oxogenic steroids.

Patient 7, aged 80 years.

Patient No. 8, *aged* 80 *years*, was admitted to hospital with slight frequency and pain on micturition. On examination the prostate was hard and the smear positive. The serum acid phosphatase was 0·4 units. X-ray examination showed osteoarthritis in the spine but no secondary deposits. Stilboestrol was administered (50 mg. b.d.) after a 6-day control period. Oestrogen excretion was very variable but in the first 8 days treatment did not affect the mean amount excreted. 17-Oxosteroid excretion fell slowly over the 8 days studied; 17-oxogenic steroid excretion increased slightly.

At the first follow-up (9 weeks) oestrogen excretion was zero and remained so at subsequent examinations. 17-Oxosteroid excretion remained depressed throughout the 85-week period of study.

At the third follow-up (27 weeks), 17-oxogenic steroid excretion was markedly depressed but rose to pre-treatment levels at 52 weeks and remained so at 85 weeks. The serum acid phosphatase was normal throughout and the patient has remained in remission.

The administration of synthetic oestrogens, therefore, generally resulted in a fall in the excretion of endogenous oestrogen either immediately or after a transient rise during the first few days of treatment. The fall was not permanent since in only one case were the oestrogen levels permanently depressed.

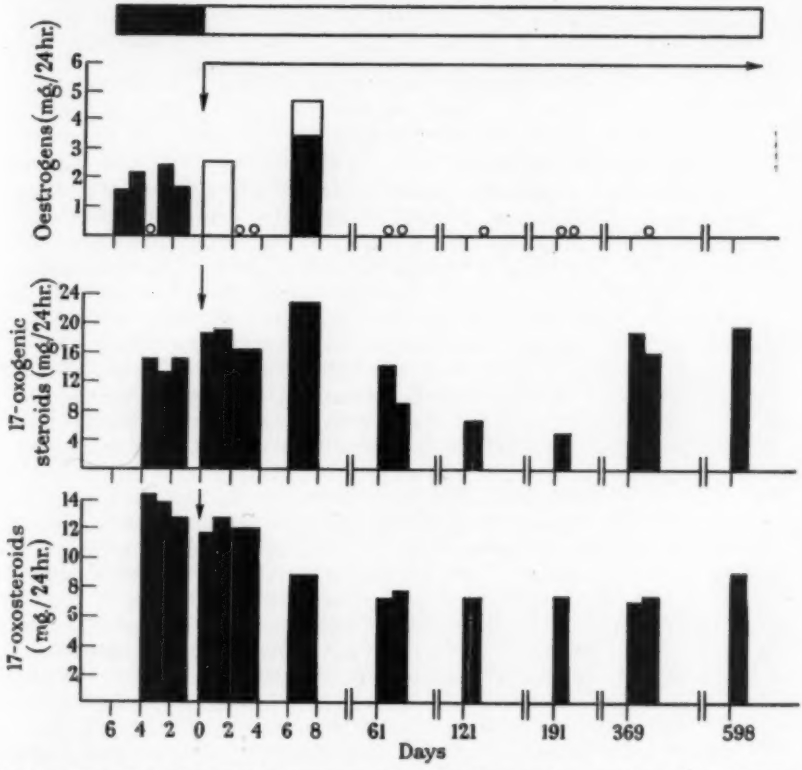

FIG. 8.—The effect of synthetic oestrogen administration on the excretion of oestrogens, 17-oxo- and 17-oxogenic steroids.

Patient 8, aged 80 years.

In this series synthetic oestrogens had little or no immediate effect on 17-oxosteroid excretion. In four cases this fraction was depressed (No. 5, 6, 7 and 8), and a minimum of 7 days treatment was required for the effect to become manifest. In the fifth case (No. 4), 17-oxosteroid excretion was unchanged over 102 weeks of treatment. In only two of the five cases was there a return to normal levels.

With the exception of Case No. 4, 17-oxogenic steroid excretion was depressed by treatment, the effect taking a week to become apparent. This fall was maintained over a long period in three patients (No. 5, 6 and 7) but in the remaining case (No. 8), excretion had risen to the control level by 52 weeks.

Oestrogen : androgen ratios

The pre-treatment oestrogen levels for the 8 patients described were divided by the 17-oxosteroid levels and the ratios obtained compared with those obtained from the data for normal controls without prostatic cancer (Bulbrook, Franks and Greenwood, 1959). There was no significant difference between the mean ratios for the two groups (0·0011 and 0·0019 respectively). In both groups the daily oestrogen/17-oxosteroid ratio was fairly constant for a particular subject but there was a 20-fold variation between individual subjects.

DISCUSSION

Changes in excretion after treatment

In a previous paper (Bulbrook, Franks and Greenwood, 1959) we have shown that the excretion of oestrogens, 17-oxosteroids and 17-oxogenic steroids in men with untreated prostatic cancer does not differ significantly from that found in patients of a similar age without clinical cancer. However after treatment with synthetic oestrogens or by castration there is a temporary fall in the excretion of one or more of the three groups of hormones measured, followed by a rise to pre-treatment levels. This biphasic response is shown most clearly by the oestrogens. The time taken for these changes to occur varies from patient to patient. Clinical improvement tends to precede marked decrease in hormone excretion, but the period of lowest hormone excretion was generally associated with retardation of tumour growth. The increase in hormone excretion to control levels again generally preceded clinical signs of renewed tumour growth.

When the results are considered in detail several further points arise. There is little doubt about the biphasic response to oestrogen treatment or to castration ; the fall and subsequent rise in excretion of at least one of the three groups of hormones is generally marked. There are two other effects which are so slight that there must be some doubt as to their general occurrence. The first of these is the slight and transient rise in 17-oxosteroids, 17-oxogenic steroids or oestrogens which sometimes occurs in the first two or three days after treatment with synthetic oestrogen is started. In the 17-oxosteroid fraction this occurred in one patient, in the 17-oxogenic steroids and the oestrogens in 2 patients. The subsequent fall in excretion was clearly manifest by about the seventh day of treatment.

The second minor effect was seen in three cases (2, 5 and 6), one a castrate, the others treated with stilboestrol. Hormone excretion fell, rose again to pre-treatment levels and then dropped again, over a period of several months. Burt, Finney and Scott (1957) found a similar change in 3 patients treated by castration. It is possible that this might have been found in other cases if specimens had been collected at shorter intervals of time. These changes did not seem to be associated with changes in the clinical state and their significance is uncertain.

The effect of castration on oestrogen excretion.—In all three cases there was a rise in excretion in the immediate post-operative period which is thought to be an adrenal cortical reaction to operative stress. A similar rise occurs after oophorectomy in the female (Bulbrook and Greenwood, 1957). Oestrogens are thought to be mainly produced by the testes in man but in two of the three cases (No. 1 and 3) castration had virtually no immediate effect on oestrogen excretion, suggesting that this hormone was being produced in some other organ, presumably the adrenal. Nevertheless, over the following three months, oestrogen excretion

slowly declined. In Case 2 however oestrogen excretion fell to roughly one-third of the pre-operative level within 14 days and there was no evidence of a further fall at subsequent examinations.

Variability of response to treatment.—The pre-treatment hormone levels found in this small series differed widely from patient to patient. Oestrogen excretion varied eight-fold between the patients, 17-oxosteroids three-fold and 17-oxogenic steroids four-fold. Although the general pattern of response to treatment was similar, the time taken for the changes to occur and the absolute amounts of hormones excreted during treatment were equally varied. This may be due to differences in response, from patient to patient, to a given dose of a synthetic oestrogen. It is possible that the most effective dose varies as widely from patient to patient as insulin requirements in diabetics. The variation in response to hormones may be genetically determined. This is well known in experimental animals and it is likely that it also occurs in man.

Not only does treatment elicit a variable response from patient to patient but in each, the three groups of hormones measured also vary independently. The oestrogens, 17-oxosteroids and 17-oxogenic steroids do not necessarily rise and fall together and in some cases only one or two of the three groups of hormones show an effect. It is probable that relative changes in excretion in the individual patient may be of greater significance than the absolute levels.

Hormone excretion and clinical state.—The similarity of oestrogen : androgen ratio in patients with and without clinical cancer suggests that a disturbance of the endogenous production of these hormones is not associated with the development of clinical prostatic cancer. However, there seems to be a rough correlation in this group of patients between the period of clinical improvement and lowered hormone excretion after treatment, particularly as far as the urinary oestrogens are concerned. This association may be fortuitous and was not invariably found in another series of cases which had been treated with synthetic oestrogens for more than five years (Bulbrook, Franks and Greenwood, 1959). All the hormones measured in this study are produced in response to stimulation by trophic pituitary hormones. If the primary endocrine disturbance associated with the growth of prostatic tumours is hypophyseal, the changes observed in steroid hormone excretion may not reflect the pituitary changes very accurately.

Another possibility which must be considered is that there is a level, perhaps varying from patient to patient, above which tumour growth is maximally stimulated. In this case the absolute amount of hormones produced would not necessarily be directly proportional to the growth activity.

Possible sources of error

In any clinical investigation a number of possible sources of error must be recognised. These include amongst others the accuracy of the original diagnosis, inadequate sample size and conscious or unconscious selection of cases. In this investigation the clinical diagnosis has been confirmed microscopically in all cases but it must be realised that the microscopy gives no guide to the biological malignancy of the tumours (Franks, Fergusson and Murnaghan, 1958). In addition only a small number of patients has been studied but the findings are in general agreement with similar investigations. In this group there has been some selection of patients, in that the severely ill patient, with urinary obstruction

or gross infection was unsuitable for investigation. Similarly, patients with small " early " tumours were often treated as out-patients and not admitted to hospital. The patients in this short-term group are therefore those with moderately severe disease but without gross urinary disturbance.

Summary and conclusions

The urinary excretion of oestrogens, 17-oxosteroids and 17-oxogenic steroids was measured in 8 patients with prostatic cancer, before and at intervals during treatment by castration (3 cases) or synthetic oestrogens (5 cases). The excretion of these hormones is depressed by such treatment but in most cases there is a later rise to pretreatment levels. This biphasic response is shown most clearly by the oestrogens. Objective regression of tumour growth in these cases, is associated with depression of hormone excretion. The later rise in excretion is generally followed by renewed tumour growth.

There is a wide variation in the response to treatment from patient to patient and each of the three groups of hormones measured may vary independently.

The changes measured may reflect changes in pituitary function. This may be the basic endocrine factor involved.

Our thanks are due to many people without whose help this investigation could not have been completed and in particular to Mr. J. D. Fergusson, Institute of Urology, St. Philip's and St. Paul's Hospitals, under whose care many of these patients were admitted ; Mr. D. Wallace, St. Peter's Hospital and Mr. G. F. Murnaghan, formerly Research Assistant, Institute of Urology.

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THE EFFECTS OF AGE ON THE STRUCTURE AND RESPONSE TO OESTROGENS AND TESTOSTERONE, OF THE MOUSE PROSTATE IN ORGAN CULTURES

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THE association between neoplasia and age is well seen in human prostatic cancer. In this disease, although the incidence increases with age, there is often a marked decline in biological malignancy (Franks, 1956), but it is uncertain whether the change is due to an alteration in the tumour or to endocrine or other changes in the host, which are known to accompany ageing. In addition the mechanism by which hormone-induced changes are produced in tumours or normal tissues in the intact animal remains obscure. By using the organ culture technique in which tissue can be maintained in an organised form *in vitro* for several weeks, it is possible to study the local tissue (or tumour) factors in isolation, and to observe the direct effects of specific hormones. There have been few reports however of studies of this type on adult tissues, except for those of Lasnitzki who, in a series of papers (Lasnitzki, 1951, 1954, 1955*a* and *b*), has described the effects of methylcholanthrene, oestrone, testosterone propionate and vitamin A on organ cultures of mouse prostate. The present paper records the effects *in vitro* of oestrogens and testosterone on the ventral prostate of mice of different ages, and is preliminary to a more detailed study of the human and rodent prostate.

MATERIAL AND METHODS

C57 mice were used in all except one series of experiments and killed by neck dislocation. The ventral prostate was removed and each lobe of the paired gland was divided into two, or occasionally, if the lobe was very large, into four pieces. Tissue from each mouse thus provided material for four or eight cultures, which were divided into four groups—one group grown on control medium and three on hormone-containing media. The cultures were explanted on rayon (4 pieces on 1 cm. squares) by Shaffer's (1956) modification of the standard watch glass technique (Fell and Robison, 1929). Each watch glass contained 2 pieces of rayon, *i.e.*, 8 cultures, on 1·5 ml. of clotted medium, composed of 0·5 ml. each of chicken plasma, chick embryo extract and human serum. The hormones used were dissolved in the human serum, to which was also added sufficient chloromycetin to give a final concentration in the medium of approximately 0·03 mg./ml. Cultures were incubated at 37° C and the medium changed every 3 or 4 days. Eight cultures from each group were fixed after 1, 3, 7, 10 and 21 days in experiments 1, 2 and 3. The media used in each of these experiments were : 1. Control medium. 2. Control medium + oestrone 2 μg./ml. 3. Control medium + oestrone 4 μg./ml. 4. Control medium + testosterone propionate 50 μg./ml.

In experiment 4, four cultures from each group were fixed after 1, 3, 7, 10 and 21 days and the media used were : 1. Control medium. 2. Control medium + oestradiol monobenzoate (O.MB.) 2·5 μg./ml. 3. Control medium + O.MB. 10 μg./ml. 4. Control medium + O.MB. 50 μg./ml.

In experiment 5, two cultures from each group were fixed after 10 days and three from each group after 17 days and the media used were: 1. Control medium. 2. Control medium + O.MB. 2 μg./ml. 3. Control medium + O.MB. 4 μg./ml. 4. Control medium + testosterone propionate 50 μg./ml.

The hormone levels given are the final concentration in the medium.

The age and strain of mice used and the number of cultures in each experiment are given in Table I.

TABLE I.—*Age, Strain and Numbers of Mice.*

	No. of mice.	Strain.	Age. (months)	No. of Cultures.
Experiment 1	38	C57	5	160
,, 2	40	C57	1½	160
,, 3	38	C57	24	160
,, 4	11	C57	4	80
,, 5	5	C3H	8	20

The cultures were fixed in Zenker's solution with 2 per cent acetic acid, embedded in paraffin wax and serially sectioned. The sections were stained with Ehrlich's haematoxylin and eosin.

Numerical assessment of results.

Since the epithelial changes to be described involved mainly the height of the cells, from base to lumenal margin, each culture was graded numerically into one of four grades (grades 1 to 4), depending on the mean epithelial height. The nuclei remained more or less constant in size and the nuclear diameter of each cell was therefore used as a measure. In grade 1, all the epithelial cells were low and the height of the cell barely exceeded the nuclear diameter. In grade 4 all the cells were tall and the height was more than twice the nuclear diameter. Grades 2 and 3 were intermediate. All the cells in one acinus were usually the same height. The acini in five sections of each culture were therefore graded according to cell height and the mean gave the numerical grade for each culture. The mean for any group of cultures gave a figure which could be used as a rough guide for comparison with other groups.

The numerical assessment of the normal gland epithelium was about grade 3.

RESULTS

The Prostate before culture—adult and young mice

The normal ventral prostate of the mouse consisted of a number of finger-like tubulo-alveolar glands opening into a common duct (Fig. 1), which opened on the ventral surface of the urethra. Each lobe of the paired gland usually consisted of two such " duct-gland " systems. In the intact organ the finger-like processes were convoluted and surrounded by a loose fibro-vascular sheath. On section (Fig. 2, 3) the larger alveoli were distended and lined by a flattened epithelium,

while the smaller generally had a number of intra-acinar papillary projections. The epithelium in the smaller acini was taller and foamy and the nucleus usually basal. Characteristically there was a supra-nuclear clear zone in the Golgi region and above this a rather dense basophil zone near the lumen. The lumenal margin of the cells was rather irregular and sometimes had a striated appearance resembling a brush border. The supra-nuclear clear zone was sometimes replaced by a few large or several small vacuoles; in actively secreting cells the whole cytoplasm had a uniformly foamy appearance. The epithelium was usually regular and only one cell thick but there were occasional basal cells. The acini each had a thin fibro-muscular coat and often contained a finely granular, slightly eosinophil secretion. The ventral prostates of the young mice (6–8 weeks), although smaller than those from older animals, had a similar histological structure, but the epithelium was generally tall and foamy resembling that of the actively stimulated gland.

Prostate before culture—old mice

The glands from these animals were smaller than those of young adults but otherwise similar to the naked eye. Histologically there were striking differences, especially in the epithelial cells which were in general lower, crowded together and often arranged in more than one layer. The intra-acinar papillae were made up of clumps of large apparently hyperplastic cells, sometimes in mitosis (Fig. 4). It is significant that mitoses are very rarely seen in the normal adult gland. The epithelial nuclei varied considerably in size and there were occasional giant cells either singly, associated with the irregular papillae (Fig. 5), or in small groups (Fig. 6). In some acini the epithelium was normal but in others, although of normal height, the vacuoles in the Golgi region were irregular in size and scattered throughout the cytoplasm (Fig. 7). The alveoli often contained small eosinophilic concretions resembling the corpora amylacea of the human prostate but without the characteristic laminations.

The muscle and fibrous tissue showed no striking changes, but there were often small collections of plasma cells in the interstitial tissue.

Changes occurring in all cultures

In addition to effects due to the added hormones, a series of irregular cellular changes appeared in all cultures. Tissues dissected and prepared as for culture and then fixed were similar in appearance to the normal intact gland but for an occasional pycnotic epithelial cell. After 24 hours' incubation, however, many cells showed a peculiar degenerative change which involved only the cells of acini at the edges of a culture; often only the outer side of an acinus was affected and not all the peripheral acini were involved. The cells were swollen and vacuolated; in some the nuclei were pycnotic, in others they appeared well preserved. In some explants the whole thickness of the epithelium showed this change (Fig 8), in others normal epithelium overlay clumps of damaged cells. After 3 days the epithelium showed great mitotic activity in areas similar to those which earlier showed degenerative changes, *i.e.*, at the edges of the culture, involving the whole thickness of the epithelium (Fig. 9) or beneath intact epithelium (Fig. 10). In some cultures, particularly if there had been severe damage involving the muscle coat also, the cellular regeneration was irregular and bizarre.

The mitotic activity in these cultures occurred at the end of a culture period before the medium had been changed, and therefore was not due to the addition of new medium.

After seven days, mitoses were very rarely seen but groups of irregular cells persisted in the cultures throughout the experiment although by 21 days they were much less prominent. In many cases the cell irregularity was very marked (Fig. 11) but the localization of the lesions, the fact that mitoses were seen only in the early stages and that similar changes appeared in cultures in all groups, suggest that the process is a regenerative one following non-lethal trauma during dissection and division of the gland lobules. This localization to an incised area is shown very strikingly in Fig. 12 which is from serial sections of the same culture as Fig. 1. The whole gland-duct system was removed by a single incision at the end of the duct. The localization of the mitotic activity to this area can be seen.

In addition to the epithelial changes there was an increase in interstitial stromal cells and sometimes of plain muscle in all cultures, although the degree of proliferation varied.

The changes described above were found in cultures in all groups, in all experiments, varying only in extent from culture to culture, but in experiment 2, using tissues from young animals, although the irregular cells were frequent at 3 days, epithelial mitoses were less common. It is probable that the period of greatest mitotic activity had occurred earlier in these cultures.

Changes probably due to added hormones

Experiment 1. *The effects of oestrone and testosterone on glands from adult C57 mice 5 months old.*—In the control cultures the epithelium retained its normal adult structure for about 10 days but after this the cells became progressively shorter, the decrease in size affecting mainly the cytoplasm. By 21 days the average height of the epithelium was about two-thirds of the normal (Fig. 13). The oestrone-treated cultures showed a greater epithelial atrophy (Fig. 14), the change appearing earlier and being more marked in the cultures treated with the larger dose. In these cultures, not only was the cytoplasm diminished but the epithelial nuclei were rather smaller and condensed. The testosterone propionate-treated cultures at 10 days showed no significant differences from the controls, except that the epithelium in some acini was rather more foamy. By 21 days the epithelium was taller than that of the normal gland with a diffusely pale-staining foamy cytoplasm (Fig. 15). The early changes were focal and appeared in only a few acini, but by 21 days almost all the acini in a culture were uniformly affected. In all groups there was a uniform increase in the interstitial stromal cells and some proliferation of plain muscle, probably more marked in the oestrone-treated cultures. A numerical assessment of the epithelial changes is given in Table II.

TABLE II.—*Epithelial Changes—Numerical Assessment. Experiment* 1

	Days			
	3	7	10	21
Control Medium (C.M.)	3·1	2·6	3·0	2·1
C.M. + Oestrone 2 μg./ml.	3·0	2·4	1·5	1·5
C.M. + Oestrone 4 μg./ml.	3·0	1·7	1·5	1·0
C.M. + Testosterone Propionate 50 μg./ml.	3·0	3·0	2·5	3·5

Experiment 2. *The effects of oestrone and testosterone propionate on glands from young C57 mice 6 weeks old.*—For the first 3 days there were no specific changes in the cultures but after this the epithelium became progressively lower in all groups. After one week the epithelium in the control and one oestrone-treated group (the other oestrone group became infected) was low and atrophic, but one culture in each group had taller epithelium. The testosterone-treated cultures also had a taller epithelium but even this was less than the normal height. By 10 days there was little difference between the groups; the epithelium was low but in all cultures occasional acini retained a normal taller type of epithelium. After 3 weeks the epithelial atrophy was more marked but the epithelium in the testosterone-treated cultures was rather more foamy than the controls though little taller than that in the other groups. All groups showed progressive increase in stroma and muscle. A numerical assessment of epithelial changes is given in Table III.

TABLE III.—*Epithelial Changes—Numerical Assessment. Experiment* 2

	Days.			
	3	7	10	21
Control Medium (C.M.)	3·0	1·2	1·75	1·5
C.M. + Oestrone 2 μg./ml.	3·0	*	1·5	1·0
C.M. + Oestrone 4 μg./ml.	3·0	1·2	1·4	1·25
C.M. + Testosterone Propionate 50 μg./ml.	3·0	2·5	1·9	2·1

* Cultures infected.

Experiment 3. *The effects of oestrone and testosterone propionate on glands from 2-year-old C57 mice.*—In general the results of this experiment showed a much greater variation in response between cultures in the same group and between acini in the one culture. Consequently a numerical assessment gives only an "average" and is therefore less reliable than from experiments 1 and 2. After 24 hours all cultures showed the traumatic vacuolation already described and by 3 days there was a similar mitotic activity in the regenerating areas (Fig. 16, 17). In all groups however the epithelium was returning to its normal adult form. The surface epithelium was taller and more regular than that of the glands before culture, giant cells were much less frequent, and the irregular clumped papillae were rarely seen. However the epithelium was still more than one cell thick in places. There were no significant differences between the control cultures and those grown on oestrone-containing media, except that the epithelium was perhaps rather lower in the cultures treated with the larger dose. By 7 days the epithelium in all cultures was lower, especially in the two oestrone-treated groups. After 10 days there were no significant differences between the control and oestrone-treated cultures. Many of the cells were low and differed little from those of the 7-day cultures, but in all there were some acini in which the epithelium was tall with supranuclear vacuoles, as in the normal adult gland. A composite numerical assessment thus gave a figure higher than that in the 7-day cultures. In the testosterone-treated cultures some cells were taller than normal and more foamy and a greater proportion of the acini were lined by such epithelium. By 21 days there was very little difference between the groups (Fig. 18–21), except that there was a greater proportion of low cells in the oestrone-

treated cultures (Fig. 19), but even in explants treated with the higher dose of oestrone some cells were still tall, with supranuclear vacuoles (Fig. 20), resembling the normal adult secretory epithelium. The testosterone-treated cultures (Fig. 21) showed little difference from the controls. In all cultures there was a gradual increase in stromal and probably muscle cells, but there was no obvious difference between the groups. A numerical assessment of epithelial changes is given in Table IV.

TABLE IV.—*Epithelial Changes—Numerical Assessment. Experiment* 3

	Days.			
	3	7	10	21
Control Medium (C.M.)	3·0	2·5	2·5	2·5
C.M. + Oestrone 2 μg./ml.	3·0	2·0	2·5	2·0
C.M. + oestrone 4 μg./ml.	2·7	2·0	2·5	1·8
C.M. + Testosterone Propionate 50 μg./ml.	3·5	2·5	2·8	2·5

LEGENDS FOR ILLUSTRATIONS

All sections are stained with Ehrlich's haematoxylin and eosin.
The magnification is × 350 except where otherwise stated (Fig. 1, 2, 16).

FIG. 1.—"Duct-gland" system of normal adult mouse prostate, 3 days culture on control medium. × 70.

FIG. 2.—Normal adult mouse prostate before culture showing small empty acini and larger distended acini. × 70.

FIG. 3.—The same at a higher magnification showing low epithelium of distended acini and tall epithelium of smaller acini.

FIGS. 4–7.—*Epithelium of prostates from 2 year old mice.*

FIG. 4.—Irregular intra-acinar papillae, with one cell in mitosis, near the top left.

FIG. 5.—Single giant cell at the base of a papilla.

FIG. 6.—A group of irregular giant cells.

FIG. 7.—Epithelium with vacuoles scattered throughout the cytoplasm, and small eosinophil concretions in the lumen.

FIG. 8.—Vacuolation and degeneration at the edge of a culture probably due to trauma, affecting the whole thickness of the epithelium. One day culture on control medium + oestrone 2 μg./ml.

FIG. 9.—Regeneration of the whole thickness of the epithelium at the edge of a culture, after 3 days on control medium.

FIG. 10.—Regeneration at the edge of a culture, beneath intact epithelium. 3 days culture on control medium.

FIG. 11.—Irregular cells at the edge of a culture present after 7 days on control medium + testosterone propionate 50 μg./ml.

FIG. 12.—Regeneration at the cut end of a duct after 3 days on control medium.

FIG. 13.—Adult prostate (5 months). Relatively low epithelium after 21 days culture on control medium.

FIG. 14.—Adult prostate (5 months). Atrophic epithelium after 21 days culture on control medium + oestrone 2 μg./ml.

FIG. 15.—Adult prostate (5 months). Tall foamy epithelium after 21 days culture on control medium + testosterone propionate 50 μg./ml.

FIG. 16.—Culture of prostate from 2 year old mouse after 3 days on control medium, showing regenerating area at the edge (right). × 70.

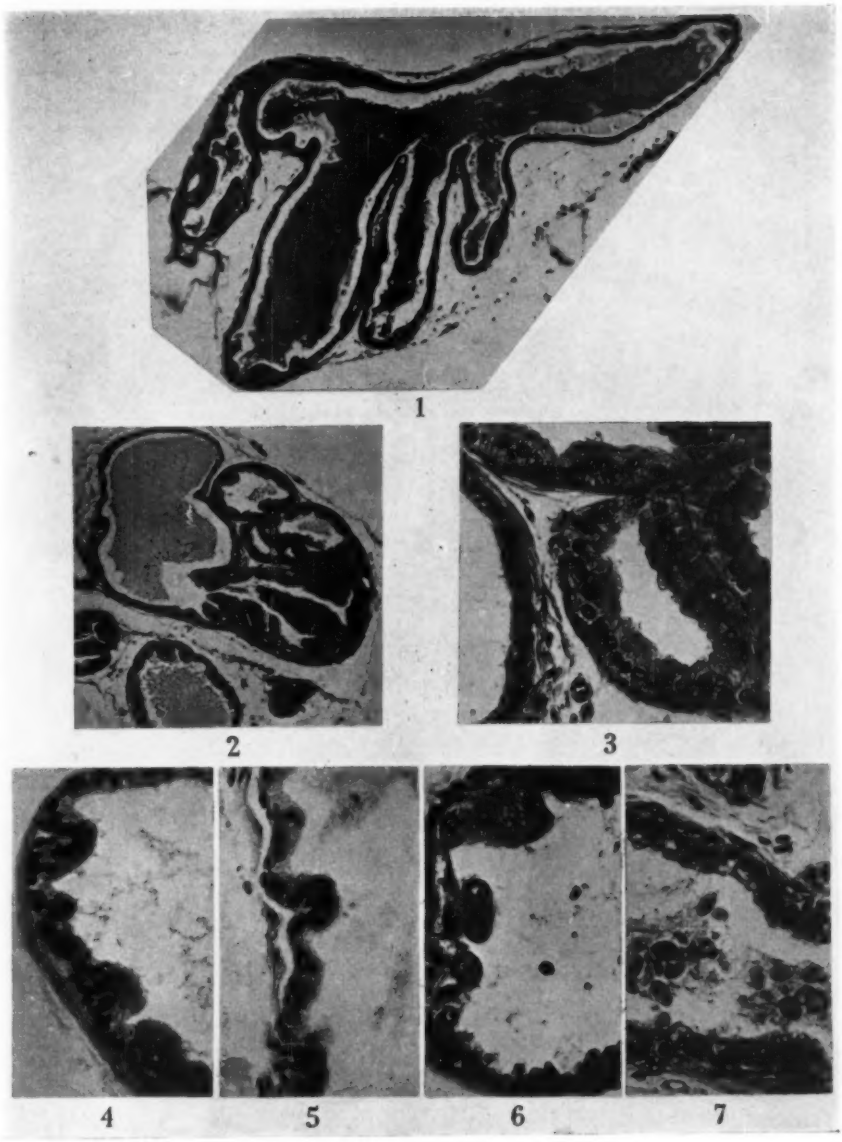
FIG. 17.—The same at a higher magnification showing regenerating area and taller epithelium resembling that of the adult gland.

FIG. 18.—Old prostate (2 years). Relatively low epithelium after 21 days culture on control medium.

FIG. 19.—Old prostate (2 years). The epithelium shows some atrophy after 21 days culture on control medium + oestrone 2 μg./ml.

FIG. 20.—Old prostate (2 years). The epithelium is tall, with supra-nuclear vacuoles in some. Twenty-one days culture on control medium + oestrone 4 μg./ml.

FIG. 21.—Old prostate (2 years). The epithelium shows little change from the control after 21 days culture on control medium + testosterone propionate 50 μg./ml.



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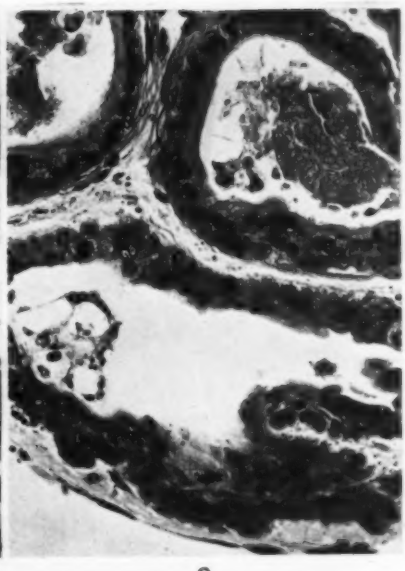

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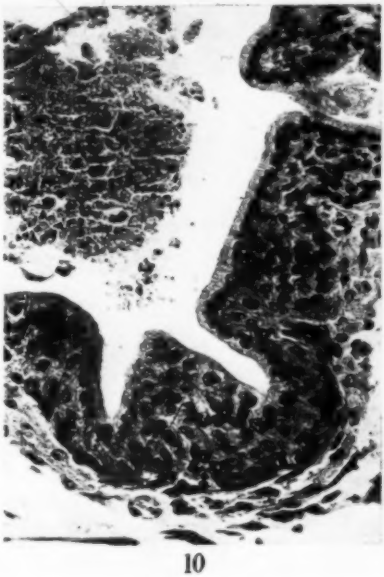

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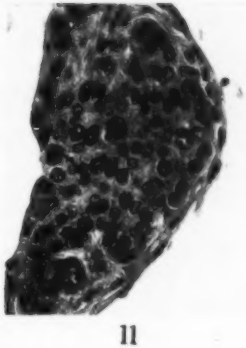

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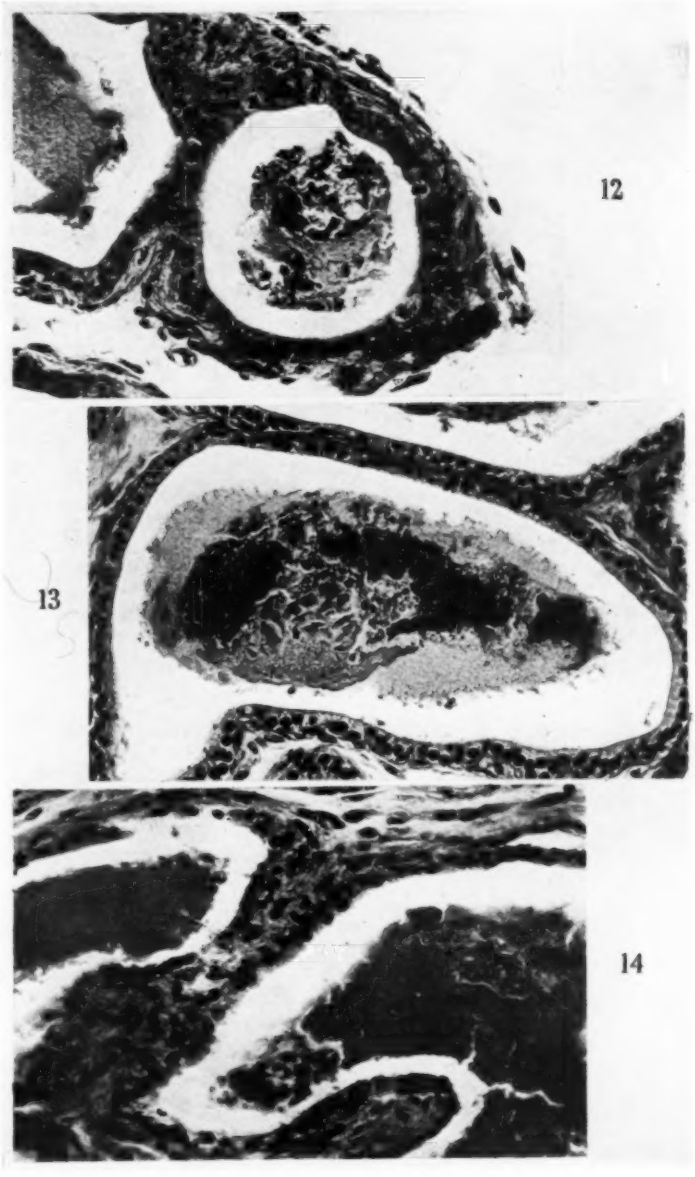

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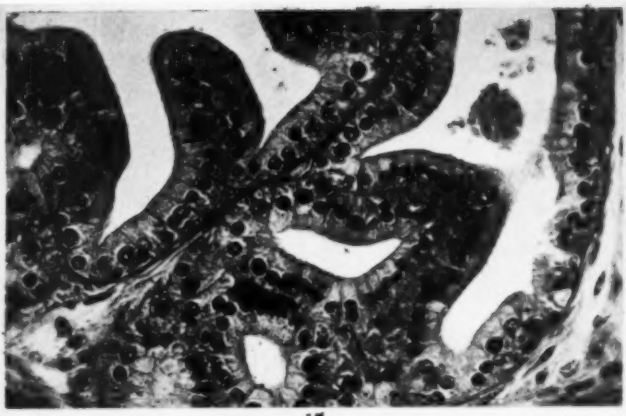

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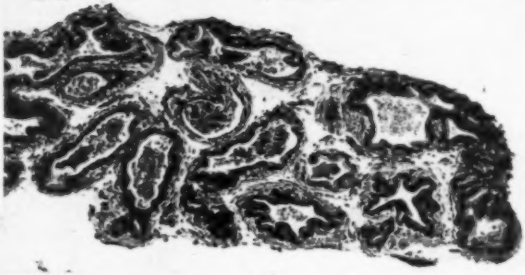

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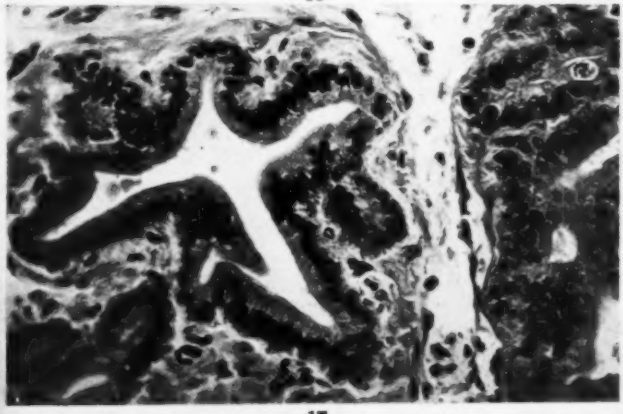

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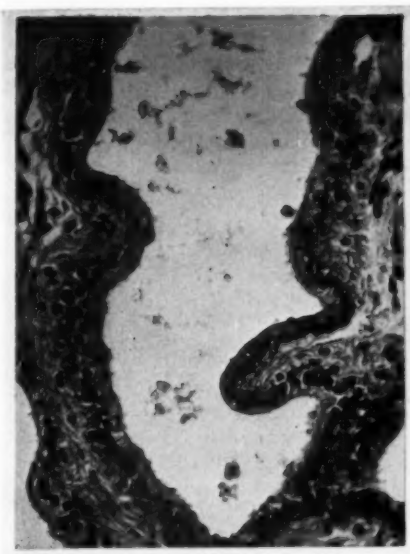

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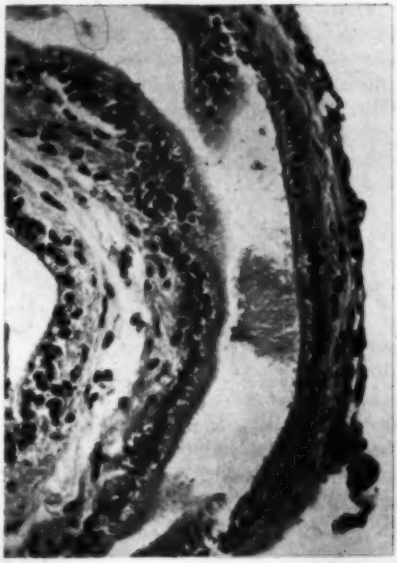

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Experiment 4. *The effects of oestradiol monobenzoate (O.MB.) on glands from adult C57 mice* 4 *months old.*—In this experiment the changes in all groups were similar, i.e., a progressive atrophy of epithelium associated with overgrowth of muscle and stroma. The epithelial changes were greater in the oestradiol-treated groups, all of which responded similarly. This suggests either that response is maximal or that the solubility of the oestradiol monobenzoate in the medium may be a limiting factor. A numerical assessment of epithelial changes is shown in Table V.

TABLE V.—*Epithelial Changes—Numerical Assessment. Experiment* 4

	Days.			
	3	7	10	21
Control Medium. (C.M.)	3·5	2·5	1·5	2·0
C.M. + O.MB. 2·5 μg./ml.	2·3	2·3	1·5	1·0
C.M. + O.MB. 10 μg./ml.	1·8	2·0	1·8	1·0
C.M. + O.MB. 50 μg./ml.	2·3	2·0	1·8	1·3

O.MB. = Oestradiol Monobenzoate.

Experiment 5. *The effects of oestradiol monobenzoate and testosterone propionate on glands from adult C3H mice* 8 *months old.*—The 10-day cultures showed no significant differences between the groups but by 17 days the epithelium of the oestrogen-treated explants was atrophic and this was more marked in the group treated with a larger dose. The control and testosterone-treated cultures both had a tall epithelium which was rather more foamy in the latter group. There was an increase in muscle and stroma, the muscular hyperplasia being particularly prominent in the later cultures treated with oestradiol monobenzoate.

Thus the responses in this experiment resembled that of the adult C57 mice. No numerical assessment was made, since there were only a small number of cultures.

DISCUSSION

The direct action of hormones on the prostate

This work confirms that of Lasnitzki, in showing that oestrone, oestradiol monobenzoate and testosterone have a direct effect on organ cultures of the prostate, resembling that seen *in vivo*, *i.e.*, epithelial atrophy and presumably secretory depression after oestrogens and epithelial stimulation after testosterone. A similar response occurred in tissue from both C57 and C3H mice. The epithelial hyperplasia and metaplasia described by Lasnitzki were not seen. This may be due to differences in responsiveness of the strain of mice used, or the composition of the medium. There was a gradual increase in stroma and muscle in all cultures and this was probably more marked in the oestrogen-treated groups.

The response to added hormones may not be due to these hormones alone, since a serum-containing medium may also have in it pituitary protein hormones as well as naturally occurring steroids. Although the levels of these hormones must be low they may still be capable of affecting the tissue response by synergistic action. There is as yet little evidence that this occurs in tissue cultures but *in*

vivo even small doses of luteinising hormone (LH) may greatly potentiate the action of follicle-stimulating hormone (FSH) as measured by uterine growth in the female (see for example Hamburger, 1950), and prolactin may have a similar effect on the response of the male prostate after treatment with luteinising hormone (LH) (Segaloff, Flores and Steelman, 1955).

There is little information about the levels of oestrogenic hormones in normal adult human male serum but it would seem to be of the order of 0·001 μg./ml. with wide variations (Preedy and Aitken, 1957). Since a response can be produced in the mouse (vagina) by the local application of oestrone in a dose of 0·00025 μg. or less (Emmens, 1950) it is possible that oestrogens present in the serum component of the medium may be sufficient to produce a tissue response especially since the strain of mouse used (C57) is known to be particularly sensitive to oestrogens (see Trentin (1950) for references). The atrophy which occurs in prostate cultures maintained on the control medium may therefore be hormone-induced. This seems a likely explanation for the finding that mouse prostates, after 6 days culture in a medium containing 40 per cent horse serum, resemble histologically those treated with oestrone (Seaman, 1956). A synthetic hormone-free medium is an obvious necessity for further work of this type, as is a more reliable indicator of cellular functional activity, since functional changes may occur without immediate change in gross morphology (Mann, Davis and Humphrey, 1949).

Differences in response to hormones at different ages.

In all groups there was a gradual epithelial atrophy in cultures maintained on the control medium, but the tissues from the old and adult animals retained their normal structure longer than those from young mice. Oestrone accelerated this atrophy, which in general appeared earlier and was more marked in those treated with the larger dose. The results of experiment 4 in which cultures were treated with large doses of a biologically more active oestrogen, oestradiol monobenzoate, suggest that this response is maximal. There was little difference in the final results of oestrone treatment on young and adult tissues but the response was earlier and more marked in the young. Tissues from the old mice however showed much less response and in some there even appeared to be secretory stimulation. The reaction to testosterone was much greater in the adult tissue but was present to a lesser extent in the explants of young tissue. Again the old tissues showed little change by 21 days, although there had been some stimulation at 10 days.

The response to both oestrone and testosterone was most consistent in adult tissue and weakest in the old. Occasional cultures of both young and old tissues differed from their groups and showed a similar reaction to that of the adult gland. Since the groups depend on the chronological age of the tissue it is likely that these exceptions had achieved an adult character earlier, or retained it longer. The relationship between chronological and physiological age is not absolute.

The young glands were much more sensitive to the depressant effects of oestrone than adult or old tissues. This may explain the greater atrophy of these glands when maintained on control medium, if this atrophy was due to oestrogens in the human serum in the medium. The relatively small response to testosterone of these young cultures is perhaps not unexpected since precocious sexual development cannot be induced by testosterone in some species. In this respect the mouse

would appear to resemble the guinea pig rather than the rat (Ortiz *et al.*, 1956; Gerall, 1958).

Changes affecting old tissues

The cellular irregularity in the prostates of old animals before culture is of particular interest. Similar changes, with giant cell formation and irregular hyperplasia have been described in other organs in old animals, e.g., liver (Andrew, 1941), bile ducts (Korenchevsky, Paris and Benjamin, 1950), pancreas (Andrew, 1944) and thyroid (Korenchevsky and Paris, 1950). In man too, with increasing age, areas of irregular hyperplasia and sometimes tumour formation have been described in a number of organs, particularly the prostate and kidney (see Franks, 1954*a* and *b* for references). Morphologically these lesions can be regarded as precancerous. Whether these changes are an intrinsic part of the ageing process in the cell or due to general factors in the host is not known. Presumably both local and general factors are concerned since if only a general factor is responsible the whole organ should be equally affected. When the tissues are maintained on control medium containing normal adult serum, however, the hyperplastic changes appear to regress and the glands tend to return to their normal adult form, thus emphasising the relative importance of the general host factor. Nevertheless these cells still differ from normal adult cells in that their responsiveness to testosterone and oestrone is greatly reduced. This may be due to deficiencies in the medium since if prostate glands from old mice are transplanted to young adult hosts the tissue may still show a secretory response. (Franks and Chesterman, not yet published).

One further point of interest is that the regenerative changes after injury in the first week of culture did not differ greatly in degree or kind in young, adult or old mice. A similar finding has been reported in a study of growth potential in regenerating rat livers (Glinos and Bartlett, 1951).

SUMMARY

Oestrone, oestradiol monobenzoate and testosterone propionate had a direct action on organ cultures of mouse ventral prostate, oestrogens causing epithelial atrophy and testosterone stimulation. The extent of these changes may be affected by other hormones present in a medium containing human serum and chicken plasma.

Tissues from young mice (1½ months) were more sensitive to the depressant effects of oestrone and less sensitive to testosterone propionate.

Tissues from old mice (24 months) were relatively less responsive to both oestrone and testosterone propionate.

The most consistent response to both hormones was obtained with tissues from adult mice (4–5 months).

All cultures showed regenerative changes in the first week of culture, probably following trauma during dissection. This reaction was similar in degree and kind in tissues from young, adult and old mice.

The prostates from old mice before culture showed areas of irregular epithelial hyperplasia which regressed after culture on media containing normal adult serum.

The possible significance of these changes is discussed.

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A SURVIVAL CURVE FOR MAMMALIAN LEUKAEMIA CELLS IRRADIATED *'IN VIVO* (IMPLICATIONS FOR THE TREATMENT OF MOUSE LEUKAEMIA BY WHOLE-BODY IRRADIATION)

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BARNES, Corp, Loutit and Neal (1956), in a preliminary report, described the successful eradication of murine leukaemia by treatment of leukaemia-bearing mice with whole-body radiation followed by intravenous isologous bone marrow. Attempts by us to cure a similar leukaemia in the same strain of mouse using similar methods and a larger dose of radiation have not been successful. It appeared that the prospects of eradicating leukaemia by this form of treatment might be usefully explored by determining a survival curve for leukaemia cells irradiated *in vivo*. Given data concerning the number of leukaemia cells in a mouse at the time of treatment, such a curve would enable the chance of cure by a specified dose of whole-body radiation to be predicted. This paper reports the determination of a survival curve of this kind. We believe that the curve will be of considerable general interest because we are not aware of a survival curve for mammalian cells irradiated *in vivo* having been described previously. An unsuccessful attempt to cure leukaemia-bearing mice by whole-body radiation is analysed in the light of the data provided by this curve.

MATERIALS AND METHODS

Mice.—The CBA mice used in all the experiments were bred by brother-to-sister mating in this laboratory and were aged 2–6 months when used for experiment.

Leukaemia strain.—The lymphocytic type of leukaemia arose spontaneously in a mouse of the same colony as that providing the mice used in the experiments, and no evidence of a genetic difference between the malignant cells and the host mice has been detected in a wide range of experiments which could have revealed such a difference. A detailed account of the leukaemia, and of the method of bioassay used to measure the number of viable leukaemia cells in a suspension, appears elsewhere (Hewitt, 1958). Briefly, the method of bioassay is as follows: a single-cell suspension of leukaemia cells is prepared from the infiltrated liver of a leukaemic mouse; the density of morphologically intact leukaemia cells is determined by counting in a counting chamber under phase-contrast microscopy, and accurate serial dilutions of the counted suspension are made; the mean number of leukaemia cells in 0·2 ml. volumes of each of the series of dilute suspensions is calculated from the cell density of the initial suspension and the dilution factor; 0·2 ml. volumes of each of a series of selected dilutions are injected intraperitoneally into a group of 6–10 mice; the incidence of leukaemia in each injected

group is recorded after a 90-day period of observation; from the results of the assay the number of morphologically intact leukaemia cells required to convey leukaemia to half a group of injected mice (the TD50) is calculated by the method of Reed and Muench (1938).

Irradiation.—To obtain data for the survival curve, individual mice were exposed to whole-body irradiation by placing them in a perforated "Perspex" cylinder closed at each end with a rubber bung, and positioning the cylinder in a beam of ^{60}Co gamma radiation from a Kilocurie beam unit (a "Theratron"). The whole-body dose was delivered at a mean dose rate of about 80r/min. and was given as equal exposures to both sides of the cylinder containing the mouse. The distance from the source to the centre of the cylinder was 62 cm. and the field size used was such as to cover the mouse very generously. Under these conditions, the whole-body dose was uniform throughout the mouse to about $\pm$ 3 per cent. Single-cell suspensions of the leukaemia cells were prepared from the livers of irradiated mice within 15 minutes of the end of irradiation and were immediately bioassayed as described. The cells were kept at 0 to 2° C. from the time of their removal from the irradiated mouse until their injection for bioassay.

For the treatment of groups of leukaemia-bearing mice, the mice were exposed (in a "Perspex"-lined metal box carrying a "Perspex" lid), to 2400r ^{60}Co gamma whole-body irradiation delivered via a single field in a single dose over a period of 22 hours. Under these irradiation conditions the whole-body dose was uniform to about $\pm$ 5 per cent. Within one hour of the end of irradiation, each of the irradiated mice received 10^6 nucleated isologous marrow cells intravenously.

EXPERIMENTS AND RESULTS

Results of Bioassay of leukaemia cells from untreated leukaemic mice

Individual mice showing signs of illness 10–14 days after their injection with leukaemia cells were sacrificed by fracture of the neck, and single-cell suspensions of leukaemia cells were prepared from the infiltrated livers and bioassayed. In 6 separate experiments the TD50 values obtained were as shown in Table I. It will be seen that no significant variation in the results of assay occurred over a considerable period of the leukaemia's history, and that the age or sex of the mice used for assay had no influence upon the end-point. The mean TD50 value for the 6 titrations, 2·0 cells, was used to signify the viability of cells from unirradiated mice in the calculation of survival rates to be described.

TABLE I.—*TD50 Values obtained in 6 separate Bioassays of Leukaemia Cells from Unirradiated Mice*

Leukaemia passage No.	Mice used for assay		Result of assay (TD50)
	Sex	Age (days)	
42	♀	96–183	1·2
49	♀	98–128	3·0
54	♂	145–172	0·7
58	♀	141–158	2·0
63	♂	184–195	3·0
70	♂	358–424	2·1

Mean TD50 = 2·0 cells

Results of bioassay of leukaemia cells from irradiated mice

In separate experiments, individual mice at the same stage of the disease as the untreated donor mice used above were exposed to prescribed total doses of whole-body radiation. Within 10 minutes of the end of irradiation the mice were sacrificed and single-cell suspensions of leukaemia cells were prepared from the livers and bioassayed. The densities of morphologically intact leukaemia cells in suspensions prepared from irradiated mice were not significantly different from those in suspensions prepared from untreated mice. On the other hand, the TD50 values obtained using cells from irradiated mice were found to be greater than the mean (2·0 cells) of the TD50 values obtained for cells from untreated mice and were a function of the dose of radiation. The survival rate in the leukaemia cell population from an irradiated mouse was calculated as follows :

$$\frac{\text{Mean TD50 for cells from untreated mice (i.e. 2·0)}}{\text{TD50 for cells from irradiated mouse}}$$

e.g. TD50 for cells from mouse which received 900r = 500

$$\therefore \quad \text{Survival rate} = \frac{2}{500} = 0{\cdot}004 = 10^{\bar{3}\cdot 6021}$$

In a series of 6 separate experiments, the survival rates among cell populations from the livers of mice which had received whole-body radiation doses ranging from

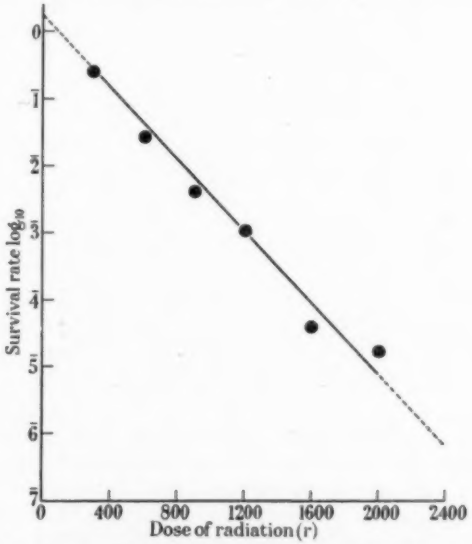

FIG. 1.—Relationship between radiation dose and log survival rate among leukaemia cells irradiated *in vivo*.

300 to 2000r were determined. The results of these experiments are given in Table II. Fig. 1 shows the relationship between radiation dose and log survival rate. Within the limits of experimental error, the relationship is linear. The line,

drawn by inspection through the points, indicates that the D_0 value (mean lethal dose of radiation, or dose required to reduce the viable cells to 37 per cent) is about 162r for ^{60}Co gamma radiation.

TABLE II.—*Survival Rate of Liver Leukaemia Cells after Specified Doses of Whole-body Radiation*

Whole-body radiation dose (r)	TD50	Fraction of cells surviving	Survival rate ($\log_{10}$)
300	8	0·25	$\bar{1}$·4
600	76	0·026	$\bar{2}$·42
900	500	0·004	$\bar{3}$·6
1200	1930	0·001	$\bar{3}$·02
1600	48,980	0·000041	$\bar{5}$·61
2000	95,500	0·000021	$\bar{5}$·32

At a later date, after the leukaemia strain had been taken through at least 12 further serial passages, two additional survival rates were determined at each of the dose levels 800r, 1400r and 2000r. All six additional survival rates were within 0·4 log of the rates predicted by the existing curve, which was thus satisfactorily confirmed.

Attempted cure of leukaemia-bearing mice by whole-body irradiation

Numerous unsuccessful attempts have been made to cure leukaemia-bearing mice with whole-body radiation followed by intravenous isologous bone marrow. In these, the following experimental conditions have been varied : size of leukaemia cell inoculum, interval between injection of cells and treatment, and total dose of whole-body radiation. The experiment to be described here combines the most favourable level for success of each of these conditions that has been employed in the previous experiments. Five CBA mice were injected intraperitoneally with a mean dose of 100 leukaemia cells. Seven to eight days after injection, the mice were exposed to 2400r whole-body ^{60}Co gamma radiation delivered in a single dose given over a period of 22 hours at a uniform dose rate. Within one hour of the end of irradiation the mice were injected intravenously with 10^6 isologous nucleated marrow cells. All the treated mice died with obvious signs of leukaemia between 14 and 18 days after irradiation. Table III shows details of the experiment including the treatment and fate of control groups. It will be seen that

TABLE III.—*Fate of Leukaemia-bearing Mice Treated with 2400r Whole-body Radiation and Bone Marrow, and of Various Control Mice*

Treatment of mice	Mortality	Survival time (days)		Mice with reticulocytes 10 days after irradiation
		After injection of leukaemia cells	After irradiation	
Leukaemia cells only	6/6	17–20 (mean, 18)	—	—
Radiation only	6/6	—	10–12	0/6
radiation + marrow	1/6	—	(5)	5/6
leukaemia + radiation + marrow	5/5	22–26 (mean, 24)*	14–18	5/5

* All these mice died with gross signs of leukaemia.

lthough none of the treated leukaemic mice survived they all had circulating eticulocytes 10 days after irradiation, indicating that the marrow grafts were unctioning, and their survival was prolonged by about 6 days compared with the ntreated control leukaemic mice. The implications of these results, considered in the light of the survival curve described above, will be discussed later.

DISCUSSION

Doses of radiation in the region of those used for determination of the survival curve for *in vivo* radiation would not be expected to have an immediate lethal effect on the leukaemia cells. This expectation has been fulfilled in our observation that the yield of morphologically intact cells from the livers of irradiated leukaemic mice is not significantly less than that obtained from untreated leukaemic mice. The radiation-induced damage with which we are concerned here is damage—most probably to genetic material in the cell—which is manifested by the cells' loss of reproductive integrity. The *in vivo* method we have used to detect cells surviving the radiation confers a precise meaning upon the term "inactivation" in the system we have used. "Inactivation" in our system means abolition of the ability of a cell to give rise to an indefinitely large population of descendants when transplanted to a healthy mouse of the same genotype as the leukaemia cell strain. If we assume that inactivation of the cells is due to a direct effect of radiation on the cells, and is not contributed to by cytotoxic constitutional influences induced in the mice by whole-body radiation (and which might continue to take a toll of the cells for many hours or days after the cessation of irradiation) then the data provided by our survival curve can be used to predict the chances of eradicating leukaemia with a given dose of whole-body radiation. The additional information required for such prediction is an estimate of the total number of leukaemia cells present in a mouse at the time of irradiation.

The survival curve here reported applies to leukaemia cells infiltrating the liver. Observations were confined to this organ because of the accuracy with which malignant cell densities can be determined in suspensions prepared from it. Single-cell suspensions prepared from such organs as the spleen contain greater numbers of non-malignant single cells, derived from the normal tissue, and the distinction of many of these normal cells from the leukaemia cells is not always reliable using phase-contrast microscopy. If we assume that the radiosensitivity of the liver leukaemia cells is representative of the total leukaemia cell population in the leukaemic mouse, it can be seen from the extrapolated part of the curve (Fig. 1) that approximately one in a million of the cells would be expected to survive a dose of 2400r. Thus, at least one million leukaemia cells of radiosensitivity comparable with that of those in the liver must have been present in the leukaemia-bearing mice at the time they were not curable by 2400r. Such a conclusion implies that the 100 cells injected intraperitoneally had given rise to 10^6 cells in the course of the 7 days elapsing between injection of the cells and irradiation. If the 100 cells injected underwent continuous logarithmic increase for 7 days, they would have to pass through about 13 generations in this time in order to reach a population of 10^6; their generation time would have to be about 13 hours. If it is assumed that the visible stages of mitosis occupy a period of 40 minutes, a population of cells undergoing reproduction with a generation time of 13 hours would be expected to display a mitotic index of about 5 per cent.

Mitotic indices at this level or higher are regularly observed in histological preparations of leukaemic livers. It can be concluded, therefore, that failure to cure the mice with 2400r whole-body radiation under the conditions described is a finding which does not conflict with our data concerning the rate of cell reproduction and the radiosensitivity of the cells as described by the survival curve (Fig. 1).

Barnes *et al.*, (1956) succeeded in curing a high proportion of CBA mice of a transplanted radiation-induced CBA leukaemia by exposing the mice to a whole-body radiation dose of 1500 rad 250kV X-rays delivered in 25 hours 7–8 days after an inoculum of 10^6 leukaemia cells. The treated mice were injected intravenously with isologous marrow soon after radiation. For the purpose of comparing the experiments of these workers with our own, we have multiplied their 1500 rad 250 kV X-rays dose by the factor 1/0·95 to express it in roentgens and by the factor 1/0·75 to allow for the relative biological effectiveness (R.B.E.) of ^{60}Co gamma rays and 250 kV X-rays, making their dose equivalent in biological effectiveness to 2105r ^{60}Co gamma rays. The factor we have applied to allow for R.B.E. is we believe, the highest that available evidence permits. (M. R. C. Unit, 1958).

It will be appreciated that the eradication of mouse leukaemia by Barnes *et al.*, using an inoculum of 10^6 leukaemia cells and irradiating the mice 7 days later with a dose equivalent to 2105r ^{60}Co gamma rays, is in striking contrast to our failure to eradicate a very similar leukaemia in the same strain of mouse using a dose of 2400r ^{60}Co gamma rays delivered at about the same dose rate and at the same interval after the injection of only 10^2 cells. From our survival curve (Fig. 1.) it is seen that a dose of 2105r is associated with a cell survival rate of $1/10^{5.4}$. Thus, at this dose level we should expect some leukaemia cells to be surviving in a mouse even in the unlikely event of the 10^6 cells of the inoculum having undergone no increase during the period of 7 days following their injection.

A comparison of the biological features of the two leukaemias revealed no striking differences, the cell type and manner of dissemination being very similar. It should be mentioned, however, that the leukaemia of Barnes *et al.* was radiation-induced and their experiments were done with material from the first few serial passages (Loutit, 1957, personal communication), whereas our own experiments were done with a leukaemia of spontaneous origin which had undergone at least 60 serial passages.

Of the many hypotheses which can be advanced to explain the apparent difference of curability between the two leukaemias, we consider that the most probable, on the limited evidence available, is that which supposes that there was some immunogenetic difference between the Barnes *et al.* leukaemia and the host mice which were treated. If this had been the case, the radiotherapy could have tipped the balance in favour of recovery of mice which were themselves developing resistance. We base this conclusion upon the considerations which follow. Firstly, in the Barnes *et al.* experiments two of the untreated control mice which were injected with 10^6 leukaemia cells failed to develop leukaemia, a finding which the authors themselves take to be evidence of "genetic drift" in the host mice. Secondly, repeated passage of a tumour, such as our leukaemia had undergone, tends to reduce the influence of slight immunogenetic incompatibility between tumour and hosts. Finally, it can be conjectured that a radiation-induced leukaemia is more likely than a spontaneous tumour to display a genetic difference from the host strain in which it is induced because it arises in an animal

with its immunological resistance partly in abeyance, due to the whole-body radiation, and because the original leukaemia cell induced may have acquired antigenic modifications from chromosomal damage done by the radiation.

It has been argued that the whole-body radiation given to the mice for treatment would have reduced their immunological resistance and thus the contribution of that resistance to cure. However, whilst whole-body radiation inhibits the institution of immunological resistance, it is known not to abolish existing immunity (Clemmesen, 1938). The radiation used for treatment would not, therefore, be expected to diminish such immunological resistance as the mice could have acquired in the 7-day period between injection of leukaemia cells and irradiation.

Cohen and Cohen (1954), after studying the influence of slight immunogenetic incompatibility between tumour and host on the radiocurability of a C3H mammary tumour concluded as follows: "It appears, therefore, that the radiosensitivity of a tumour is a quantitative measure of subliminal host-resistance resulting from immunogenetic differences in the host-tumour relationship, including extra-chromosomal factors".

SUMMARY

Using a quantitative method for the transplantation of mouse leukaemia cells within a closely inbred colony of CBA mice, it was found that an average of 2 cells was required to transplant leukaemia to half a group of injected mice (i.e. TD50 = 2 cells). When cell suspensions were transplanted from donor mice soon after their exposure to whole-body radiation, the TD50 was found to be greater and to be a function of the radiation dose. From the data of several experiments of this kind, using different doses of radiation, a radiation dose-log cell survival rate curve was determined for the leukaemia cell population irradiated *in vivo*. The implications of the curve, which was linear, did not conflict with the results of experiments in which unsuccessful attempts were made to cure leukaemia-bearing mice by their exposure to whole-body radiation followed by the injection of isologous bone marrow. The successful cure of a similar CBA leukaemia by Barnes *et al.* (1956) using similar methods is discussed in relation to data presented here.

We are grateful to Miss J. J. Howarth (B.E.C.C. Research Physicist) for carrying out all the irradiations done using the "Theratron", and to Mrs. D. Levy for skilled technical assistance in relation to the biological work. We are grateful also to the British Empire Cancer Campaign for a whole-time research grant to one of us (H.B.H.) and for financial support to the laboratory of the other (C.W.W.).

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THE EARLY DEVELOPMENT OF TUMOURS IN MICE INOCULATED WITH A CELL-FREE FILTRATE OF MOUSE LEUKAEMIC TISSUE

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GROSS reported the frequent occurrence of leukaemia in mice of the low-leukaemic strain C_3H inoculated when newborn with centrifugates or filtrates of normal or leukaemic tissue from the high-leukaemic strain AK. When inoculated C_3H mice were mated before the onset of leukaemia, 13 out of 27 untreated progeny developed the disease (Gross, 1951*a* and *b*). Epithelial tumours of the neck, apparently arising in salivary glands, and other tumours of diverse types, also appeared in inoculated mice (Gross, 1953*a* and *b*). A few such tumours arose in C_3H mice inoculated with centrifugates of normal C_3H organs (Gross, 1955*b*).

Considerable differences in susceptibility to leukaemogenesis were found between substrains of C_3H. The Bittner substrain proved the most susceptible, and within this substrain mice not carrying the milk factor were at least as susceptible as those that carried it (Gross, 1957*a*). Other donor and recipient strains were used, with results which differed in detail (Gross, 1954, 1956*a*).

Centrifugates or filtrates of induced salivary gland tumours, sarcomata, and other tumours, produced either leukaemia, or tumours, or both, in varying proportions when injected into newborn mice (Gross, 1957*a*).

Treatment of leukaemic tissue extracts with ether destroyed their leukaemogenic activity but not their power to produce salivary gland and other tumours (Gross, 1956*b*). Heating at 60°C for 30 minutes had a similar effect; 68–70°C for 30 minutes was required to abolish tumour-producing power (Gross, 1953*b*). High speed centrifugation (144,000 × g.), filtration through candles of fine porosity or Gradocol membranes of 93–140 mm. A.P.D., or even simple dilution (Gross, 1955*a*), all reduced leukaemogenesis without appreciably affecting tumour production (Gross, 1957*a*; Latarjet, 1958). There were also marked differences in relative frequency of the two effects in recipient mice of different strains (Gross, 1954) and between mice of the same strain inoculated at different ages (Gross, 1958*c*).

Since 1951 experiments similar to those of Gross have been carried out in many laboratories. In some (e.g. Woolley and Small, 1956; Dulaney, 1956) very similar results have been obtained, in others Gross' observations have been confirmed in part (e.g. Law, Dunn and Boyle, 1955; Stewart, 1955*a* and *b*; Furth *et al.*, 1956). In all these experiments, as in those of Gross' the absence of tumours at the sites of injection, the long latent periods, and the fact that leukaemic extracts gave rise to tumours and vice versa, make it very unlikely that the effects were due to a few intact cells in the inocula. Direct evidence has been given by Dulaney *et al.*, (1957) and by Latarjet (1958) that no viable leukaemic cells remain after grinding and centrifugation, in either supernatant fluid or deposit. Moreover in the great majority of those cases where donor and recipient differed antigenically it was

possible to show by transplantation that the induced leukaemias and tumours were of recipient type.

Before 1951 virtually all attempts to transmit mouse leukaemia by cell-free inocula had failed. One report (Engelbreth-Holm and Frederiksen, 1938) of a success was not confirmed (see Engelbreth-Holm, 1942, for older literature). Since 1951 also there have been a number of negative reports, notably in this country (e.g. Powell and Pearson, 1954, 1955; Stern, 1956; Elek, Jewell and Stern, 1957; Weavers, Powell and Pearson, 1957; Hewitt, 1956, 1958). In this laboratory cell-free preparations of a carcinogen-induced transplantable leukaemia in mice of the 101 strain have been injected by various methods into newborn mice of the same strain, without result. These unsuccessful experiments have all differed in some point of method or materials from those of Gross, but those which succeeded also varied widely in these respects.

Meanwhile several related phenomena have been described which indirectly support Gross' contentions. Leukaemic extracts from a high leukaemic strain greatly accelerate the onset of leukaemia in mice of the same strain inoculated when newborn (Rudali, Duplan and Latarjet, 1956, 1957), and this effect has been used as a method of assay by Latarjet and his colleagues in a remarkable series of investigations (see Latarjet, 1956, 1958, for review). The potency of the agents in extracts of leukaemic or salivary gland tumour tissue have been raised by serial passage through newborn mice (Gross, 1957*b*) or tissue cultures (Stewart *et al.*, 1957, 1958), and the latter treatment has been shewn to render them active in another species (Eddy *et al.*, 1958*b*). Myeloid leukaemia has been induced by Graffi and his colleagues in several strains of mice inoculated when newborn, or up to 11 days old, with cell-free filtrates of the Ehrlich carcinoma, and of other transplantable tumours (e.g. Graffi *et al.*, 1955; Graffi, Fey and Bielka, 1956; Graffi, 1957). Friend (1957*a* and *b*) has also isolated a filtrable agent from the Ehrlich carcinoma, which rapidly induces a monoblastosis indistinguishable from reticulum-cell sarcoma (Furth, 1959), when injected into adult mice.

There is evidence which suggests that under suitable conditions these agents may have haemolytic or haemagglutinating action (Gross, 1948; Eddy *et al.*, 1958*a*), recalling the fact that extracts of many mouse tumours have been shewn to have similar effects on erythrocytes of various species (Gross, 1948; Salaman, 1948). Recent attempts to determine which structural or chemical component of the cell is associated with leukaemia or tumour-inducing action are still at an early stage. Latarjet (1958) has given evidence that a cytoplasmic fraction without the mitochondria is the most active, and there is electron-microscopic evidence which is consistent with this (Dmochowski and Grey, 1957; Graffi, 1957). It has been suggested that some activity may be carried by nucleic acid alone, though this is by no means proved (Hays, Simmons and Beck, 1957; Latarjet, Rebeyrotte, and Moustacchi, 1958; Graffi, 1957). However nucleoprotein precipitates are highly active (e.g. Bielka, Graffi and Krischke, 1957; Fjehlde, 1958).

Interpretations of these phenomena have differed widely. Gross regards his observations as indicating the action of several viruses (e.g. Gross, 1951*b*, 1957*a*, 1958*b*). These, he believes, are normally transmitted through the embryo, and are capable of remaining latent for long periods, i.e. from a few months to several generations in high-leukaemic strains, and much longer—sometimes indefinitely—in low-leukaemic strains and strains in which the tumours induced by inoculation

rarely if ever occur spontaneously. Others have drawn attention to analogies with bacterial transformation, the transduction of bacterial characters by bacteriophages, and the activation of prophages. The greater susceptibility of newborn mice has suggested comparisons with the induction of immune-tolerance. Several reviews of fact and theory have appeared in recent years (e.g. Furth *et al.*, 1956 ; Latarjet, 1956, 1958 ; Graffi, 1957 ; Gross, 1957*a*, 1958*a* and *b*).

Much remains obscure, and some important results require confirmation with larger numbers of animals and more extensive controls. No explanation or analogy so far advanced appears wholly satisfactory, and it may be that a type of induction of neoplasia has been encountered which is new in our experience.

The experiments to be described formed part of a series undertaken not merely as a repetition of those of Gross and others, but with the object of obtaining a potent cell-free leukaemia-inducing or tumour-inducing agent for future study.

Tumours (but no leukaemia) have appeared in unexpectedly large numbers, after short latent periods, in mice inoculated when newborn with a cell-free filtrate of leukaemic tissue. The methods used were based on those of Gross (1953*b*). Spontaneous leukaemia in mice of the high-leukaemic AKR strain provided the source material, and mice of the low-leukaemic C_3H (Bittner) substrain were the recipients. The characteristics of both strains are well known here and elsewhere. Particular attention was paid to the exclusion of viable cells from the inoculum.

MATERIALS AND METHODS

AKR mice were derived from a litter obtained from the Christie Hospital and Holt Radium Institute, Manchester in September, 1957. Spontaneous leukaemia of lymphatic type has arisen in more than half of these AKRs between 8 and 12 months of age.

C_3H (Bittner) mice, carrying the milk factor, were derived from a litter obtained from the Imperial Cancer Research Fund Laboratory in 1953. The strain was received by them in 1941 from the Royal Cancer Hospital, London, who got it from Dr. Bittner at Bar Harbor in 1938. No leukaemia, nor any tumours other than hepatomata and mammary carcinomata, have been observed here in untreated mice of this strain.

Mice were bred by brother-sister mating.

Mice received from other laboratories were vaccinated on arrival with sheep lymph (Lister Institute) as a precaution against mouse pox (Salaman and Tomlinson, 1957). Breeding mice were vaccinated at 3 weeks of age. The C_3H mice inoculated with leukaemic filtrate, as described below, were not vaccinated. Mice were kept in metal boxes, and fed a cubed diet based on the Rowett Institute formula (Thomson, 1936) and water *ad libitum*.

Lemco broth was obtained from Oxo Ltd., and made up 10 per cent in Ringer's solution. Oxoid membranes were also obtained from Oxo Ltd. Gradocol membranes were supplied by Dr. Himmelweit of the Wright-Flemming Institute, St. Mary's Hospital.

EXPERIMENTAL PROCEDURE

An 8 months old AKR male, one of the original breeding litter received from Manchester, developed visible enlargement of axillary and inguinal lymphatic glands, and was killed. There was enlargement of the thymus (12 mm. diameter),

and all abdominal lymphatic glands (mesenteric 15 × 4 × 4 mm.); the spleen was deep red in colour, not grossly enlarged, but with a broad lower pole and a finely dimpled surface. Other organs appeared normal to the naked eye. Sections of liver, lungs, and kidneys shewed widespread infiltration with cells of the lymphoid series. These appearances are typical of the spontaneous leukaemia which arises in 60–85 per cent of AKR mice, though there is considerable variation in the degree of involvement of different organs.

A cell suspension made from spleen, thymus, and lymphatic glands was injected into adult AKR mice, and they developed a similar leukaemic condition in 13 days. After three passages by injection of cell suspension, the spleen, lymphatic glands, and thymus of a mouse injected 17 days previously were thoroughly ground in a Ten Broeck glass grinder, with the addition of 16 ml. cooled Lemco broth. This and subsequent manipulations were carried out in a cold room at + 2°C. The suspension was spun in a Sorvall angle centrifuge (Model SS1) at 5000 r.p.m. (3000 × g.) for 15 minutes. The supernatant fluid was removed and the deposit resuspended in a further 16 ml. of Lemco broth, and spun again. The pooled supernatants were filtered through an Oxoid membrane, and then through a Gradocol membrane of APD 0·42 μ, at negative pressures of 100–300 mm. Hg. The final filtrate, in which the tissue dilution was approximately 1 : 30, was stored in glass ampoules in a solid CO_2 box. A freshly-grown culture of *Bact. coli* was then filtered through the same Gradocol membrane, and the filtrate added to McConkey's broth. No growth was seen during 6 days incubation.

On the day following filtration an ampoule of filtrate was thawed, and immediately 0·03 ml. was injected subcutaneously into the scapular region of each of 15 C_3H mice less than 17 hours old (one litter of 7 and another of 8). A week later another ampoule was thawed, and 13 more C_3H mice less than 17 hours old (a litter of 7 and a litter of 6) were injected similarly. Of the four inoculated litters 5, 5, 7, and 6 mice, respectively, survived until the first tumours appeared (11 weeks).

RESULTS

The development of tumours in inoculated mice

Table I gives details of the tumours which have appeared during the first 6 months of observation. It will be seen that 15 out of 23 surviving mice in the 4 inoculated litters have developed 40 tumours. All the 15 tumour-bearing mice belong to the first 3 litters (17 mice). No mouse has yet developed leukaemia.

C_3H mice of comparable age, belonging to previous and subsequent litters, have been under observation. No tumours have been seen in them.

Naked-eye and microscopic examination of the tumours

Tumours of the neck consisted of bunches of firm grey nodules, 1 to 10 mm. in diameter, partly replacing normal salivary gland tissue. They were often bilateral, lightly adherent to surrounding tissues, and sometimes extended as a collar round the neck and forwards into the buccal and temporal regions (Fig. 1). A contiguous but clearly distinct presternal or axillary mass was often seen.

Microscopical appearances were similar to those previously described (e.g. notes by Dr. M. N. Richter and Dr. J. Furth in Gross, 1953*a*; Law, Dunn and Boyle,

1955). The predominant cell was large, polygonal, rapidly dividing, with pale basophilic cytoplasm and large pale-staining nucleus. The histological structure varied widely both between tumours and within the same tumour. All the tumours appeared multifocal, consisting of well-defined, roughly spherical lobules. The cells were often arranged in acinar and tubular groups in the periphery, and in undifferentiated masses or bundles in the centre, of the tumour lobules (Fig. 2); other lobules consisted solely of undifferentiated cells. In some, broad bands of eosinophil hyaline material separated the cells. The tumour lobules were interspersed with, and sometimes imbedded in apparently normal lobules of the salivary glands. Areas of hyperplastic duct tissue were found (Fig. 3), and a continuous series could be traced in some tumours from these to the frankly neoplastic areas (Fig. 4).

The commonest tumours of the trunk were subcutaneous, soft, grey, usually lobulated masses, lightly adherent to skin and body wall. They varied much in size (5 to 25 mm. diameter), and often contained large pockets of necrotic material. Three of these tumours occurred in males (mice 1, 2, and 9) and eleven in females (mice, 3, 4, 5, and 12) (Fig. 5).

Microscopically the predominant cell was similar to that in the less differentiated parts of the neck tumours. The histological structure varied, even within the same tumour. Anaplastic cell masses, often split up by endothelium-lined clefts, were found close to groups of more or less well-formed acini and cysts (Fig. 6), some containing milk-like secretion. Tumours in males (Fig. 7) did not differ essentially from those in females.

In two females bearing tumours of this kind, grossly hyperplastic, probably precancerous, mammary glands were found (Fig. 8).

These tumours were considered to be carcinomata. Their origin is discussed below.

Four tumours of different type arose. A small tough white subcutaneous tumour of the abdominal wall of mouse 18, and similar larger tumours of the flank and scapular region respectively in mice 19 and 22, all firmly adherent to

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EXPLANATION OF PLATES

FIG. 1.—Male mouse 9 showing salivary gland tumours.

FIG. 2.—Lobule of salivary gland tumour showing differentiated peripheral, and undifferentiated central, area. Note many mitoses in latter (♂ 3) × 110.

FIG. 3.—A group of hyperplastic ducts in an otherwise normal salivary lobule, at the edge of a salivary gland tumour (♂ 8) × 110.

FIG. 4.—Larger areas of hyperplastic duct tissue, adenomatous lobules, and the edge of an undifferentiated carcinomatous mass (♀ 22) × 110.

FIG. 5.—Female mouse 3, killed at 18 weeks, showing tumours in both inguinal regions (the left shows scar of biopsy), and axillae.

FIG. 6.—Part of a subcutaneous carcinoma in the inguinal region of a female mouse (♀ 3) × 170.

FIG. 7.—An adenocarcinoma from the inguinal region of a male mouse (♂ 9) × 14.

FIG. 8.—Hyplerplastic, probably precancerous, mammary tissue in a female mouse bearing a carcinoma of the inguinal region (♀ 12) × 13.

FIG. 9.—Fibrosarcoma invading the muscular wall of the abdomen (♂ 18) × 220.

FIG. 10.—Emboli of undifferentiated tumour tissue in the lung (♂ 9) × 385.

FIG. 11.—Cortex of kidney showing an area of cellular infiltration (♂ 2) × 250.

FIG. 12.—A high-power view of the same kidney showing a dilated hyperplastic tubule and adjoining area of cellular infiltration (♂ 2) × 475.

Fixative: Zenker-acetic.

Stains: Ehrlich's haematoxylin and eosin.

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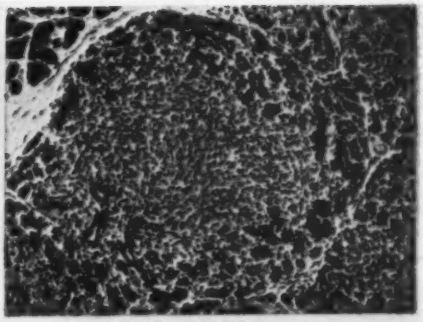

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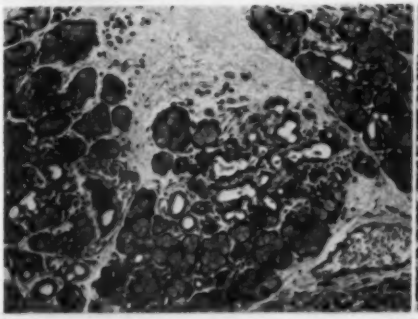

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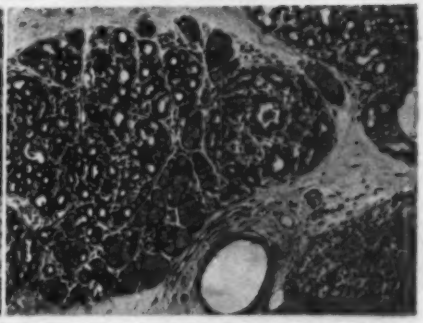

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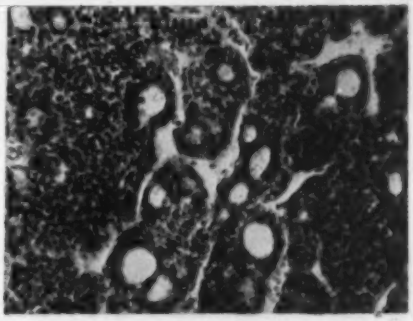

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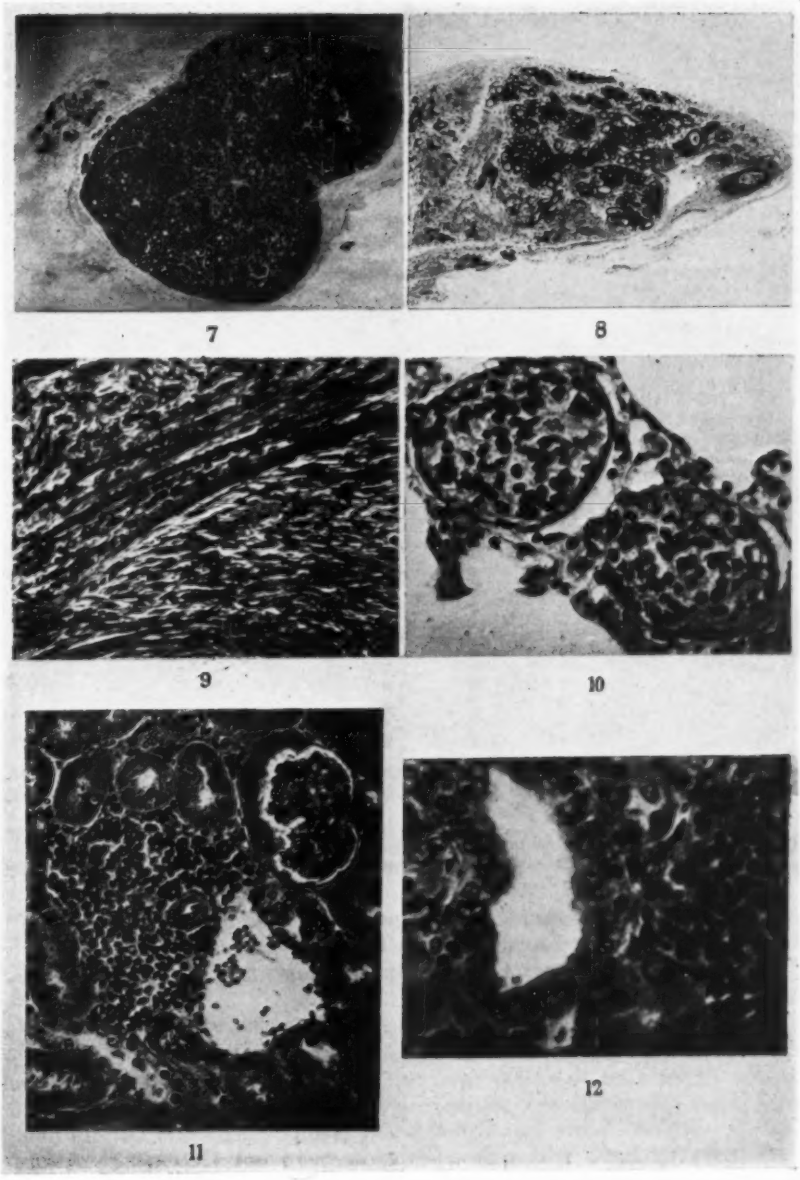

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11 12

TABLE I.—*Tumour-Incidence up to 29 Weeks after Inoculation of Newborn C_3H Mice with a Gradocol Filtrate of AKR Leukaemic Tissue*

Litter No.	Mice alive when 1st tumour appeared	Age when 1st tumour appeared (weeks)	Site of 1st tumour	Age when killed (weeks)	Total tumours	Tumours of neck*	Tumours of trunk	Histological type of tumours of trunk	Figures
I	♂ 1	11	Trunk	15	1	0	1	Adenocarcinoma	
	♂ 2	15	Neck	20	3	2	1	Undifferentiated carcinoma	11, 12
	♀ 3	11	Trunk	18	6	2	4	All were mixed adeno- and undiff. carcinomata	2, 5, 6
	♀ 4	15	„	16	4	2	2	Adenocarcinomata	—
	♀ 5	15	„	20	4	1	3	All were mixed adeno- and undiff. carcinomata	—
II	♂ 8	19	Neck	24	2	2	0	—	3
	♂ 9	18	„	26	3	2	1	Adenocarcinoma	1, 7, 10
	♀ 10	15	„	17	1	1	0	—	—
	♀ 11	15	„	Alive	2	2	0	—	—
	♀ 12	15	„	24	4†	2	2	Both were mixed adeno- and undiff. carcinomata	8
III	♂ 16	19	Trunk	25	3	2	1	Fibromyxosarcoma	—
	♂ 17	21	Neck	26	2‡	2	0	—	—
	♂ 18	20	Trunk	21	1	0	1	Fibrosarcoma	9
	♂ 19	23	„	29	1	0	1	„	—
	♂ 20	—	—	Alive	0	0	0	—	—
	♀ 21	—	—	„	0	0	0	—	—
	♀ 22	19	Neck	28	3	2	1	Fibrosarcoma	4
IV	♂ 23	—	—	Alive	0	0	0	—	—
	♂ 24	—	—	„	0	0	0	—	—
	♀ 25	—	—	„	0	0	0	—	—
	♀ 26	—	—	„	0	0	0	—	—
	♀ 27	—	—	„	0	0	0	—	—
	♀ 28	—	—	„	0	0	0	—	—
Totals	23 mice				40	22	18		

* The numbers 1 or 2 in this column signify unilateral and bilateral involvement of the salivary glands, respectively. These tumours were multicentric; no attempt was made to count individual foci. For histological description see text p. 80.

† Also a hyperplastic mammary gland; see text p. 80 and Fig. 8.

‡ Found dead: post mortem changes too advanced for histological study. In addition to the neck tumours, there was a mass (6 mm. diameter) in the region of the left adrenal gland (not included in tumour count).

skin and deep tissues, proved to be spindle-cell sarcomata (Fig. 9). A large, grey, gelatinous, translucent, firmly adherent subcutaneous tumour of the flank in mouse 16 was a myxosarcoma with spindle-cell areas.

Invasive properties of the tumours

Except for the 4 tumours last mentioned, which freely infiltrated surrounding tissues, including muscle (Fig. 9), the invasive power of both trunk and neck tumours was low; suprisingly so considering their sometimes very rapid growth and high mitotic rate. They spread *en masse* through areolar and fibrous tissue, but muscle planes were not penetrated. The skin over them ulcerated late, if at all. Lymph glands adjacent to, or embedded in, the neck tumours were often replaced by growth. Intravascular tumour emboli were found in the lungs of

male mouse 9 (Fig. 10). This mouse had bilateral, predominantly undifferentiated, salivary gland tumours, and a well-differentiated secreting adenocarcinomatous mass in one inguinal region (Fig. 7). On histological grounds it was thought unlikely that the latter was a metastasis from the former. The emboli in the lung however, probably came from the salivary gland tumours.

No other distant metastases were found.

General condition of tumour-bearing mice, and changes in organs

All the tumour-bearing mice, except two, remained in good health, even when bearing very large tumours. The two exceptions (mice 1 and 18), each with a small tumour of the abdominal wall, suffered progressive wasting and had to be killed at the 15th and 21st week respectively. In mouse 1 this happened in spite of the successful removal of the tumour 3 days after it was first seen. Post mortem both mice shewed atrophy of lymphatic organs. The spleens shewed decrease of lymphoid tissue, and thickening of the capsule and trabeculae. The condition of these mice, and the appearance of their spleens, was reminiscent of that described by Law and Dunn (1951), which was ascribed by them to a transmissible infective agent carried by, but separable from, a transplanted leukaemia. In the other tumour-bearing mice no significant changes were observed in liver, lungs, spleen, thymus, lymph glands, or adrenal glands. Naked-eye examination of the bony skeleton, intestinal tract, heart, and male and female sex organs, revealed no abnormalities.

On the other hand the kidneys of all tumour-bearing mice shewed well-defined changes. Masses (20 to 300μ diameter) of small round cells and plasma cells, with a few mitoses, were found around small arterioles in the cortex (Fig. 11). Often but not always in close relation to these masses, abnormal tubules, consisting of large pale-staining epithelial cells, were seen. They were usually dilated, and were strikingly different in appearance from the convoluted tubules of the normal cortex (Fig. 12). These structures were not seen in normal C_3H mice, but a few small masses of round cells, similar to those described above, have been found in the kidneys of normal C_3H mice of similar age. Stewart *et al.* (1957, 1958) have described these dilated tubules in kidneys of mice bearing tumours induced by tissue culture fluids infected with leukaemic extracts. They noted the collections of round cells, which they also regarded as infiltrations by lymphocytes and plasma cells.

Transplantation of induced tumours

Tumours obtained at biopsy or post mortem were minced and implanted with trocar and canula, or injected as coarse tissue suspensions in Ringer's solution, subcutaneously into young adult C_3H and AKR mice. When a graft grew, further transplants were made from it into mice of the same strain. The results are given in Table II. Grafts of carcinomata from mice 3 and 5 have grown in a proportion of C_3Hs, but not in AKRs. None of the 3 neck tumours grew in either C_3Hs or AKRs. Four sarcomata have been transplanted recently: one shews early growth (2 weeks).

It is not unusual for spontaneous mammary carcinomata of C_3Hs to grow somewhat irregularly in early transplants. Their growth often improves in subsequent passages, and this may be happening with the two transplantable

TABLE II.—*Transplantation of Induced Tumours*.

Inoculated mouse	Tumour	Transplant	Grafted mice† AKR	Grafted mice† C_3H
♂ 1	Carcinoma (body wall)	1st	0/4	0/8
♀ 3	„ „ „	„	0/8	5/12
	„ „ „	2nd	—	10/10
		3rd	—	1/4
♀ 4	„ (neck)	1st	0/4	0/8
♀ 5	„ (body wall)	„	0/4	5/12
		2nd	—	6/8
		3rd	—	2/3
♂ 9	„ (neck)	1st	—	0/8 (2 days old)
♀ 10	„ „	„	0/4	0/8
♀ 12	„ „	„	—	0/8
♂ 16	Myxosarcoma (body wall)	„	—	1/4
♂ 18	Fibrosarcoma „ „	„	—	0/8
♂ 19	„ „ „	„	0/4	4/4
♀ 22	„ „ „	„	0/4	0/4

† Number of takes/number of grafted mice.

filtrate-induced tumours of this type in the present series. Their invasive power has increased after transplantation. Salivary gland tumours often fail to grow when transplanted: Law, Dunn and Boyle (1955) succeeded with 7 out of 15 attempted. Others also report variable results (Furth *et al.*, 1956). Further attempts will be made to transplant these tumours. The salivary gland tumour from mouse 9 was implanted subcutaneously into eight 2 day old C_3H mice, but there has been no sign of growth so far (5 weeks).* It is noteworthy that no leukaemia has so far developed in AKR mice grafted with any of these tumours.

DISCUSSION

The results now reported add to the existing evidence for the presence of a tumour-inducing agent in cell-free extracts of mouse leukaemic tissues.

The tumours which arose in this series were not essentially different in type, distribution, number, or time of appearance from those described in comparable series by others. No mouse has developed leukaemia, but 6 months is a short time for this, and those mice which survive may still do so. The tumour incidence (40 tumours among 15 out of 23 inoculated mice) was as high, and their latent period as short, as in any comparable series in which material not previously passed through tissue cultures or newborn mice has been injected. The fact that all tumours have appeared so far in 3 litters (17 mice) and none in the 4th (6 mice) is curious, but the occurrence of non-susceptible litters is well recognised (e.g. Law, Dunn and Boyle, 1955).

Apart from the absence of tumours in the fourth litter, other differences between litters were apparent. The average latent period of first tumours was 13½ weeks in the first litter, 16½ in the second, and 20½ weeks in the five tumour-bearers of the third. In the 1st and 2nd litters all tumours of the trunk were carcinomata, in the third litter all were sarcomata. The third and fourth litters

* This tumour grew in two of these mice between the 10th and 12th weeks.

were inoculated after the filtrate had been removed from the freezing box for the second time, with a week's storage intervening. Whether this influenced the results it is impossible to say.

The site, clinical course, and histological appearance of the tumours must be considered in conjunction with those described by others. The authors responsible for the most detailed histological study of these tumours yet made (Law, Dunn and Boyle, 1955) were very cautious in their classification.

The subcutaneous carcinomata of the trunk shewed widely varying degrees of differentiation, but they were not demonstrably different from the spontaneous mammary tumours of C_3H mice.

Those which arose in females in the present series would have been unhesitatingly described as mammary carcinomata, were it not that very similar tumours were seen in males. This does not of course exclude a mammary origin but suggests caution before it is accepted. Mammary tumours in male mice except under the action of oestrogen are practically unknown, and no other signs of oestrogen action in the inoculated male mice were noted.

The possibility was considered that the carcinomata of the trunk in males might be metastatic deposits from salivary gland tumours. This could not be excluded on histological grounds alone, but was thought improbable for two reasons. Firstly it is very unlikely that metastases would be confined to a few distant subcutaneous sites. Secondly, in one case (male 1) a large secreting adenocarcinoma on the abdominal wall developed very early (11 weeks), and no salivary gland tumours were seen, either then, or when the animal was killed a month later. (The apparently normal salivary glands were not sectioned).

The neck tumours were certainly epithelial, and multicentric. Because of the occurrence in their vicinity of areas of hyperplasia of the ducts of the serous salivary glands it seems probable that this was their tissue of origin.

No tumours of this type have arisen spontaneously in our C_3H (Bittner) mice. Tumours of the neck have been seen occasionally in mice of the 101 strain, and in (101 $\times$ CBA) F_1 hybrids (the latter had received applications to the skin of 9 : 10-dimethyl-1 : 2-benzanthracene and croton oil). These tumours were very variable in microscopical appearance, and though they contained elements similar to those found in the induced neck tumours of the present series their structure was quite different. They shewed no sign of multicentric origin, and were in general much less differentiated. They are being further studied.

In spite of the conspicuous differences between the carcinomata of neck and trunk which have been described and illustrated, areas were often found in each which could have come from the other, and these were not only in the least differentiated parts. It is tempting to speculate about the indication of these similarities, but the close proximity of mammary and salivary gland tissue in the neck of the mouse should not be forgotten. Some of the neck tumours may well have been of mixed origin.

The sarcomata were of well recognised histological types.

SUMMARY

1. Mice of the C_3H (Bittner) substrain were inoculated subcutaneously when less than 17 hours old with a cell-free filtrate of a homogenate of leukaemic tissue from a mouse of the AKR strain.

2. Forty tumours appeared in 15 out of 23 inoculated mice which survived for 3 months or more. The first tumour appeared after 11 weeks, the latest after 23 weeks.

3. Twelve mice bore subcutaneous tumours of the neck, almost all bilateral. They were multicentric carcinomata. Appearances suggested origin from salivary duct epithelium.

4. Eleven mice bore one or more subcutaneous tumours of the trunk. Of these tumours 14 resembled mammary carcinomata, 3 in males and 11 in females. There were also 3 fibrosarcomata, and one fibromyxosarcoma.

5. Metastasis to the lungs was observed in a mouse bearing tumours of mammary and salivary gland types.

6. Four salivary gland tumours, 3 carcinomata of mammary type, and 4 sarcomata, were implanted into both C_3H and AKR mice. Two mammary tumours and 1 sarcoma grew in C_3Hs. No tumour grew in AKRs.

7. Large collections of inflammatory cells, often associated with hyperplastic tubules, were seen in the cortex of the kidneys of all inoculated mice so far examined. No significant changes were seen in other organs.

8. No leukaemia has so far developed in any of the inoculated or grafted mice.

I am much indebted to my colleague Dr. F. J. C. Roe for his help in the histological examination of the tumours, to Drs. L. Foulds, W. Jacobson, and G. Landells who have given me the benefit of their special experience, and to Dr. Edith Patterson, and Prof. J. Craigie for the gift of breeding litters. I am also grateful to Messrs. W. J. Milton, A. L. Stiff, C. Choong, J. Chapman, and Mrs. J. Cohen, for technical assistance.

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THE EFFECT OF APPLYING CROTON OIL *BEFORE* A SINGLE APPLICATION OF 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA).

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THE effect of giving croton oil before treatment with a carcinogenic hydrocarbon has been studied previously by others (Berenblum, 1941; Mottram, 1944*a*, 1944*b*; Berenblum and Shubik 1947; Berenblum and Haran, 1955). Mottram claimed that pretreatment with croton oil increased the tumour yield obtained from 3 applications of 3,4-benzpyrene followed by further croton oil treatment. Berenblum and Shubik failed to confirm this and considered that croton oil only promotes tumour development when it is given *after* treatment with a carcinogenic hydrocarbon. Mottram produced a few malignant tumours in his experiments, but Berenblum and his colleagues have confined their studies to the induction of benign tumours. In the present paper the effect of reversal of order of treatments is examined primarily with regard to its possible effect on the incidence of malignant tumours.

Previous studies (Roe, 1956*a*) have established that malignant skin tumours appear in "S" strain mice several months after a single application of 9,10-dimethyl-1,2-benzanthracene (DMBA), and that the incidence of such tumours is considerably and significantly increased by an 18-week course of applications of croton oil begun shortly *after* the DMBA. A study of benign tumour development and subsequent regression in the same mice led, however, to a less clear-cut result. Mice which received DMBA once followed by croton oil quickly developed many papillomas on the treated area, but more than half of them regressed before the mice died (i.e. by about 65 weeks). In these mice only a few tumours appeared outside the treated area. Mice treated with DMBA only, on the other hand, developed few papillomas on the treated site but many outside it, mainly on the face and head. These appeared late and were for the most part still present at the time of death. Several explanations could be put forward to explain the occurrence of the ectopic tumours in mice painted with DMBA only, but it is difficult to see why the mice painted with DMBA and croton oil did not develop a similar, or even higher, number of such tumours.

The present paper describes an experiment in which three groups of "S" strain mice were given either DMBA once only, DMBA once *followed by* an 18-week course of croton oil, or DMBA once *preceded by* an 18-week course of croton oil.

MATERIALS AND METHODS

The materials and methods used were essentially the same as those described in the first paper of this series (Roe, 1956*a*).

One possibly important difference was that the "S" strain mice used in the present experiment were born and reared in metal cages in this laboratory,

whereas those used in the earlier experiment were imported at the age of 5–7 weeks from an animal dealer who houses his mice in non-creosoted wooden cages.*

EXPERIMENTAL

Fiftyfour male and 27 female "S" strain mice were vaccinated on the tails with sheep lymph against ectromelia, and divided at random into three equal groups each of 18 males and 9 females. At the start of the experiment the mice were 6 to 7 weeks old. The dorsal hair was removed by electric clippers before the start of the experiment and subsequently when necessary.

Group 1 were given a single application to the clipped dorsal skin of 0·2 ml. 0·15 per cent DMBA in acetone, and no further treatment.

Group 2 were given a similar application of DMBA, and then, starting 3 weeks later, a course of 18 once-weekly applications of 0·3 ml. croton oil in acetone. The concentration of croton oil was 0·1 per cent for the first two weeks and thereafter 0·17 per cent.

Group 3 were given 18 once-weekly applications of croton oil followed, after a 3-week interval, by a single application of DMBA.

All mice were examined regularly for skin tumours throughout their lives. Those with large malignant skin tumours were killed and specimens taken from the tumours for histological examination. The experiment was terminated at 69 weeks, by which time more than half the mice had died or been killed.

RESULTS

The results can be analysed in two ways, depending on whether the beginning of treatment or the time of application of DMBA is chosen as the common starting

* Mice born and reared in wooden cages which had previously been painted with cresote as a preservative had a higher incidence of lung adenomas than mice of the same strain born and reared in metal cages (Roe, Boutwell and Bosch, 1958). Moreover such mice reacted differently to croton oil treatment (Boutwell and Bosch, 1958). It is not yet known whether contact with creosote was entirely responsible for the difference, or whether the wooden cages themselves were partly to blame.

TABLE I.—*Tumour-incidence* 23, 46, *and* 69

Group	Treatment	Sex	Number of Mice	23 Weeks Benign tumours: Survivors	Mice with papillomas	Total papillomas on treated area	Total papillomas outside treated area	46 Weeks Benign tumours: Survivors	Mice with papillomas	Total papillomas on treated area	Total papillomas outside treated area
1	DMBA only	M	18	16	0	—	—	13	2	0	2
		F	9	8	0	—	—	8	0	—	—
2	DMBA followed by croton oil	M	18	16	13	67	0	13	5	16	4
		F	9	8	7	40	0	8	6	20	0
3	DMBA preceded by croton oil	M	18	17	1	2	0	14	7	3	20
		F	9	9	1	1	0	9	4	3	5

point for the comparison of tumour incidence. Table I shows tumour development in relation to the time of application of DMBA, tumour incidence being shown at 23, 46, and 69 weeks respectively, after DMBA. If the results shown for group 3 in the 23-week column are shifted to the 46-week column, and those in the 46-week column to the 69-week column, it becomes possible to compare the results at 46, and at 69 weeks after the beginning of the experiment.

There are objections to either method of comparison. A single application of DMBA is a far more potent carcinogenic stimulus than a single application of croton oil. This is clear from previous experimental results (Roe 1956*a*, 1956*b*). Therefore if the start of treatment is taken as the common point then group 3 is handicapped in that it received this strong carcinogenic stimulus 21 weeks later than the other 2 groups. Alternatively, if the time of application of DMBA is taken as the starting point, group 3 has the advantage of a few papillomas which would be expected from treatment with croton oil alone. From previous work (Roe 1956*b*), however, it is known that " S " strain mice treated with 18 weekly applications of croton oil and then kept under observation without further treatment develop only a few benign tumours during treatment and no further tumours when treatment is stopped. Moreover, such mice do not develop malignant skin tumours. After taking these facts into account it was thought more valid to compare the results taking the time of application of DMBA as common for the 3 groups.

Whichever method of comparison is used it will be apparent that group 3 is not strictly comparable with the other two groups because DMBA was given when the mice were 26–27 weeks old instead of 6–7 weeks old.

In Table I benign and malignant tumour-incidence are recorded separately for the skin of treated area and for that outside the treated area. All the tumours recorded as " malignant " showed penetration of the panniculus carnosus muscle as seen histologically. The tumours classified as " probably malignant " had many of the characteristics of malignancy including active infiltration of the dermis, but did not show invasion of the panniculus muscle.

69 *weeks after application of DMBA*

46 Weeks						69 Weeks								
Malignant tumours						Benign tumours				Malignant tumours				
Mice with malignant or probably malignant tumours	On treated area		Outside treated area			Survivors	Mice with papillomas	Total papillomas on treated area	Total papillomas outside treated area	Mice with malignant or probably malignant tumours	On treated area		Outside treated area	
	Malignant	Probably Malignant	Malignant	Probably Malignant							Malignant	Probably Malignant	Malignant	Probably Malignant
0	—	—	—	—	.	8	3	3	6	0	—	—	—	—
0	—	—	—	—	.	4	2	0	3	0	—	—	—	—
3	4	1	0	0	.	6	2	2	1	3	4	1	0	0
3	3	2	0	0	.	4	1	3	2	3	3	2	0	0
1	0	0	1	0	.	—	—	—	—	—	—	—	—	—
1	0	0	0	1	.	—	—	—	—	—	—	—	—	—

DISCUSSION

Let us first consider the tumours which arose on the treated area. Twenty three weeks after the application of DMBA mice which received DMBA followed by croton oil (group 2) had numerous papillomas on the treated site, whereas those which received DMBA preceded by croton oil (group 3) had very few. This finding confirms Berenblum and Haran's (1955) observations. At the same time mice treated with DMBA only (group 1) had no tumours. At 46 weeks there were still no tumours on mice of groups 1, the number on mice of group 3 had risen slightly but all were benign. On the other hand, in mice of group 2 despite considerable regression of papillomas, there were several malignant tumours. After a further 23 weeks a few benign tumours had appeared in group 1, more had regressed from group 2, and no more malignant tumours had arisen. No figures are available for group 3 at 69 weeks. The differences between groups 1 and 3 are probably not significant, and one may conclude quite confidently that as far as the treated site is concerned, croton oil promoted tumour formation when given after a single application of DMBA but not when given before it. The promoting effect was evidenced by the rapid production of large numbers of papillomas which subsequently regressed, and by the appearance of malignant tumours.

The position with regard to tumours arising outside the treated area (ectopic tumours) is both different and less definite. There were no ectopic tumours in any of the groups at 23 weeks. By 46 weeks there were 25 benign and 2 malignant tumours in group 3,and only 2 and 4 benign tumours in groups 1 and 2, respectively. At 69 weeks the incidence of ectopic tumours was higher in group 1 than in group 2. Taken by themselves, these results suggest that croton oil given before DMBA increases the incidence of ectopic tumours but that croton oil given after DMBA decreases it. This suggestion however should be accepted with caution. Firstly because in a previous experiment with " S " strain mice (Roe, 1956*a*) the incidence of ectopic tumours 46 weeks after treatment with DMBA only (e.g. group 1) was actually higher than that in group 3 in the present experiment (65 on 38 survivors, all benign). Secondly, Berenblum and Haran (1955) produced more ectopic tumours by DMBA followed by croton oil than by DMBA alone. In the latter case it must be emphasised that many differences exist between the techniques used by Dr. Berenblum and his colleagues and those used in this laboratory (e.g. strain of mice, solvent, volume and frequency of dose, duration of treatment and observation etc.).

It is not possible to be sure therefore, whether in the present experiment the carcinogenic effect of DMBA was in anyway enhanced by croton oil treatment given beforehand, but it is certain that, at least as far as malignant tumours are concerned, the enhancement was less than when the same course of croton oil is given afterwards. It does not necessarily follow however that croton oil would under all experimental conditions have less enhancing effect when given before DMBA than when given after it. The pattern of experiments of which the present is an example, was first laid down by Mottram (1944*a*) and has become, since the classical experiments of Berenblum and Shubik (1947), almost a ritual. It should be remembered that this pattern includes careful choice not only of test tissue and species but also of size and frequency of dose (that of the initiator so that it produces few if any tumours alone, and that of the promoter so that it will be

tolerated for a prolonged period). In the present test the order of administration of the two treatments was simply reversed. A more stringent test of the effect of reversal would be to apply a single large dose of croton oil, followed by repeated very small doses of DMBA, or other " initiator ". If practically possible this might give a different result from that reported here.

SUMMARY

1. An experiment is described in which three comparable groups of mice were treated once with DMBA, once with DMBA and then 18 times with croton oil, or 18 times with croton oil and then once with DMBA. The incidence of papillomas at 23 weeks, 46 weeks, and 69 weeks, and the cumulative incidence of malignant tumours, in the 3 groups is compared.

2. Only the group which received DMBA before croton oil developed malignant tumours on the treated site.

3. Mice which received DMBA after croton oil developed more tumours than those treated with DMBA only, but as in the latter, most of the tumours (including 2 malignant ones) were outside the treated area.

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OIL OF SWEET ORANGE: A POSSIBLE ROLE IN CARCINOGENESIS

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OIL of sweet orange is obtained by expression from the peel of *Citrus sinensis*. It is imported into Great Britain from a variety of countries, but mainly from the U.S.A. The oil, or an essence prepared from it, is used as a food additive especially by the soft drinks, confectionery, and bakery trades; it is also used pharmaceutically. The main constituents are limonene (> 90 per cent) and decanal. Citral, methylanthranilate, linalol and terpineol are present in some samples but not all, and there are probably yet further compounds present which have not so far been identified. These may include substances used to spray fruit against insect or other pests. The main constituent, limonene, is a monoterpene and does not contribute to the flavour of the oil. Decanal is the constituent mainly responsible for flavour.

The experiment reported here was part of a research project in which certain essential oils were to be tested for carcinogenic or co-carcinogenic activity for the skin of mice. In a preliminary test several oils (turpentine, lemon, peppermint, cinnamon, cloves, cedar wood) were applied to the skin of mice and biopsies taken 3 days later. Oil of sweet orange (B.P.C. grade), applied neat, was found to cause considerable epidermal hyperplasia. Moreover, the hyperplasia could be made to persist indefinitely and without concomitant ulceration by weekly applications of the neat oil or of an 80 per cent solution of the oil in acetone. None of the other oils tested gave rise to such a marked hyperplastic response. Oil of orange was therefore selected for test for carcinogenic and co-carcinogenic activity.

Forty male and 40 female mice of the " 101 " inbred strain maintained in this laboratory were divided into 4 groups of 20 mice each consisting of 10 males and 10 females. The first group were given a single application of 9,10-dimethyl-1,2-benzanthracene (DMBA), 300 μg. in 0·2 ml. acetone to the clipped dorsal skin, and no further treatment. The second group were given once weekly applications of 0·25 ml. neat oil of sweet orange (00). The third group received a single application of DMBA followed, after a 3 week interval, by weekly applications of neat 00. The fourth group were similar to the third except that instead of neat 00, weekly applications of 20 mg. urethane dissolved in 0·25 ml. 80 per cent 00 in acetone were given as the second treatment. The reason for the use of urethane in the 4th group is not relevant here, however it should be pointed out that previous results indicated that weekly applications of 60 mg. urethane in acetone after a single application of DMBA did not lead to tumour formation in " S " strain mice (Salaman and Roe, 1953).

The experiment is in its 24th week and all the mice are still alive. No papillomas are present in groups 1 or 2. In group 3, 9 mice have a single papilloma each;

and in group 4, 8 mice have a total of 15 papillomas. New papillomas are still appearing, and those already present are increasing in size. Seventeen out of the 24 are 2 mm. or more in diameter and the largest is 8 mm. In addition 1 mouse of group 3, and 1 of group 4 have developed haemangiomatous tumours in the subcutaneous tissue of the treated area. No malignant tumours of epidermal origin have so far been seen.

The experiment is being repeated and extended, and discussion will be deferred until the results of these further tests are known.

SUMMARY

" 101 " strain mice painted with DMBA once followed by oil of sweet orange weekly developed many papillomas of the skin and one subcutaneous haemangioma. The addition of urethane to the oil of orange slightly increased the yield of tumours. Mice treated with DMBA only, or oil of orange only, developed no tumours during the same period. No malignant skin tumours have arisen so far (24 weeks).

The expenses of this research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

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ADDENDUM.

Since this paper was submitted for publication, malignant skin tumours have arisen in two mice of group 4, at 23 and 26 weeks respectively. On histological examination one of these tumours was found to be an undifferentiated carcinoma invading muscle: the other has not yet been examined histologically.

STUDIES OF THE CARCINOGENIC ACTION OF TRICYCLOQUINAZOLINE

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TRICYCLOQUINAZOLINE (TCQ) was synthesised by Cooper and Partridge (1954) during investigations on cyclic amidines. This compound is built up of three benzene rings and three pyrimidine rings fused together in such a way as to yield a fully symmetrical structure (Fig. 1). As far as is known, it is unrelated to any of the homocyclic or heterocyclic carcinogenic hydrocarbons hitherto studied and is characterised by unusually high chemical stability. It is, for example, resistant to oxidation and is stable to sublimation at red heat.

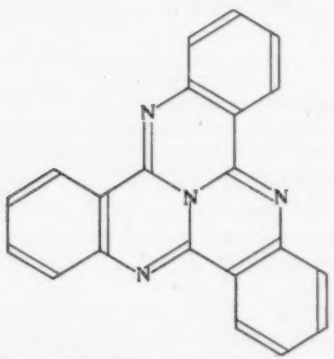


FIG. 1.—Tricycloquinazoline.

More recently, it has been shown that TCQ is readily formed in simple pyrolytic reactions with a number of derivatives of anthranilic acid, including methyl anthranilate (Baldwin *et al.*, 1958). In view of the wide occurrence of methyl anthranilate in plant materials (Klein, 1932) and the ease of production of TCQ from it by combustion, it was considered essential to determine the carcinogenic activity of TCQ. In a preliminary report (Baldwin *et al.*, 1958) TCQ was shown to possess weak but unusual carcinogenic properties following subcutaneous injection into rats. The present paper is concerned with a detailed report of the carcinogenic activity of TCQ administered subcutaneously in rats and by skin painting in mice.

MATERIALS AND METHODS

Subcutaneous injection

The compound was injected subcutaneously into male and female inbred Wistar rats (7 to 11 weeks old) as a suspension (10 mg./ml.) in sterile olive oil, B.P. Injections were made by inserting the needle through a sterilised portion of the cervical region and pushing it down into the right axilla. This prevented leakage of the TCQ suspension.

Initially, it was intended to give monthly injections of 10 mg. of the compound. However, the material was found to persist subcutaneously at the site of injection and therefore only 4 injections were given (total dosage per animal of 40 mg.). Animals were examined weekly throughout the period of the experiment (26 months) and at the conclusion, all remaining animals were sacrificed and the TCQ cysts removed for histological examination.

Skin painting

Mice.—Stock albino mice (Schofield strain) of both sexes and 8 to 10 weeks old were used. These were obtained commercially and maintained on a cubed diet (M.R.C. diet 41) supplemented with fresh greenstuffs and carrots, plus water *ad libitum*.

Technique

Tricycloquinazoline was prepared as a saturated solution (1 mg./ml.) in double re-distilled A.R. grade benzene. This solution was freshly prepared every two to three weeks and kept tightly stoppered in all-glass bottles in the dark.

The hair was removed from the whole back of the animal with electric clippers at the beginning of the experiment and subsequently when necessary. The solutions were applied dropwise from all-glass tuberculin syringes, care being taken to ensure even spreading over an area of approximately 4 cm. $\times$ 3 cm.

Forty mice, equally divided with respect to sex, were skin painted twice weekly with 0·3 ml. of the TCQ solution (0·1 per cent w./v.) for 40 weeks. An equal number of mice were treated with benzene to serve as controls. Mice were examined, for the presence of tumours, twice weekly during the period of treatment and the numbers seen were charted. Following completion of the skin-painting treatment, all survivors were examined weekly until they died or were killed with tumours. Mice were killed when it was considered that tumours were apparently malignant. The position of all tumours was recorded and sections of the apparently malignant tumours were taken for histological examination. Malignancy of tumours was based on such observations as rapid enlargement, extensive ulceration, and invasion of neighbouring structures.

RESULTS

Subcutaneous injection in rats

Tricycloquinazoline proved to be extremely resistant to metabolism and subcutaneous cysts containing the compound remained at the site of injection for the duration of the experiment (22 to 26 months). Quantitative data on the metabolism of TCQ are not yet available, but attempts were made to recover the compound 22 months after injection from two rats which had large cysts.

In each case, approximately 36 mg. out of the 40 mg. injected were recovered from benzene extracts of the cyst fluids. The recovered compound was shown to be identical with TCQ by comparison of melting points, mixed melting points, ultra-violet absorption spectra and R_F values (Butler, 1958).

Tumours developed in 8 out of 30 rats (27 per cent) which survived for 12 months or longer after the first injection. The first tumour appeared 14 months after the beginning of the experiment, whilst the majority arose between the 16th and 24th months. All these tumours arose in association with the TCQ cysts (Fig. 2, 3). TCQ cysts were found in all rats that died as well as in the animals remaining (8) at the completion of the experiment (26 months). In some cases, the cysts were single and relatively large, whilst in others they were multiple and proportionately smaller. From an examination of about 50 sections of TCQ cysts it was possible to identify various stages in the evolution of the tumour. The inert compound, which was visible in histological section by fluorescence microscopy, was surround by a fibrous wall which showed some variations in thickness. In animals without tumours, this fibrous tissue appeared inert. In some of the younger lesions, a granulomatous reaction with giant cell formation was seen (Fig. 4).

Histological examination of tumours illustrated their close relation to the cyst, which was often intact (Fig. 2) and in some places the tumour could be seen closely blended with the cyst wall (Fig. 5). In all cases, tumours were cellular sarcomas, probably of fibrous origin, and their irregularity and frequent mitotic figures left no doubt as to their malignancy (Fig. 6).

Skin painting in Mice

Four of the mice treated with TCQ died within 60 days. The remaining 36 mice survived throughout the experiment or developed tumours and were killed. Papillomata first began to appear after 110 days (average tumour induction period 189 days) and by the time skin painting was completed (280 days) 26 mice (72 per cent) had developed tumours (average tumour yield 5·2). The great majority of the tumours developed in the area painted, though a few occurred on the abdomen and face of the animals.

As the nature of these latter tumours was in every way similar to those arising in the skin painted area it appears likely that they arose by transfer of TCQ to the face and the abdomen by the animal.

The tumours appeared first as papillomas which were mostly of the sessile variety. Ulceration soon followed and was often accompanied by infection

EXPLANATION OF PLATES

FIG. 2.—The thoracic wall is exposed and shows a tumour mass lying in the subcutaneous tissue, the animal's head being on the right of the photograph. A small cystic space, which was found to contain TCQ, can be seen below the main tumour mass.

FIG. 3.—Low-power view to show relation of TCQ to tumour mass. × 12.

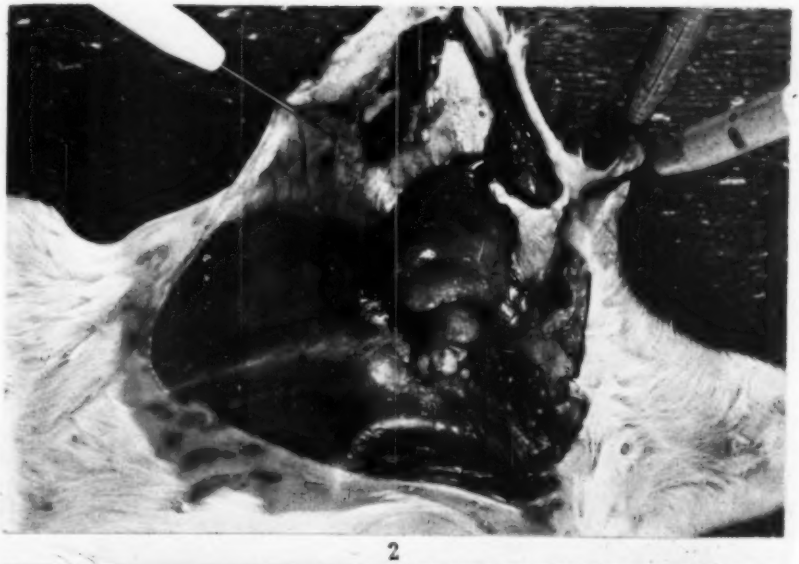
FIG. 4.—A portion of the cyst wall showing histiocytic and giant-cell reaction to the foreign substance. × 110.

FIG. 5.—High-power view of cyst and related cellular tissue. × 85.

FIG. 6.—Irregularity of neoplastic elements and presence of numerous tumour giant cells. × 225.

FIG. 7.—Large ulcerated skin tumour in mouse resulting from painting with TCQ.

FIG. 8.—Low-power view of section of entire tumour shown in Fig. 7. The general structure of the lesion resembles that seen in keratoacanthoma in man. × 4·5.



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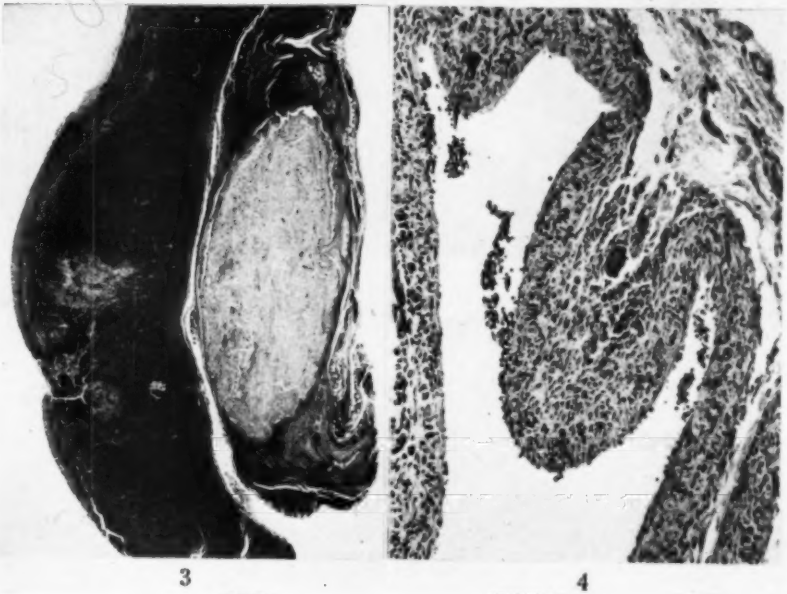

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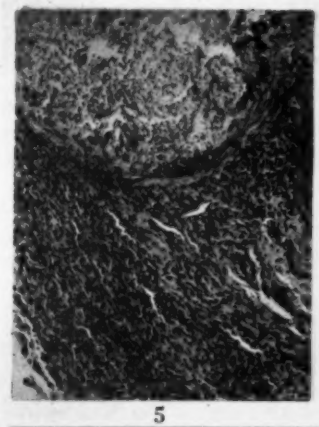

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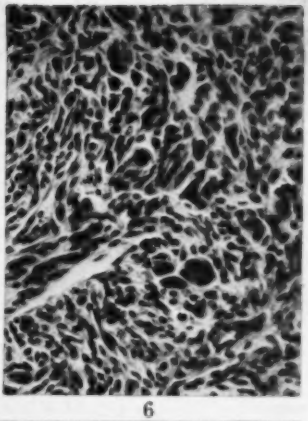

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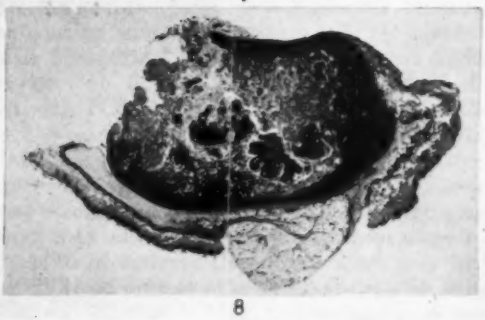

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(Fig. 7). Section of an entire tumour showed it to be composed of a central ulcerated area with a central mass of keratin (Fig. 8). This was surrounded by a area of proliferating and active squamous epithelium in which much cellular irregularity and many mitotic figures could be seen. This appearance was in every way similar to the histological lesion of keratoacanthoma in man, which is known to retrogress spontaneously. In a number of animals the skin painting was stopped to see whether the tumours would persist. Not only did they persist, but extension of growth occurred and the neoplastic tissue in some cases was seen to invade neighbouring structures. Malignant tumours were observed in 24 out of 36 mice at risk. Most of these showed the characteristic histological appearance of squamous cell carcinoma with varying degrees of keratinisation. A few tumours were less differentiated and the histological features were those of basal cell carcinoma.

In the control benzene-painted group of 40 animals one developed a small simple papilloma on the back

Discussion

The present findings confirm that tricycloquinazoline is carcinogenic for mouse skin and rat subcutaneous tissue, although the activity of the compound varies considerably with the route of administration. Thus skin painting induces a high incidence of tumour formation with a short latent period comparable with that shown by many other skin carcinogens. In contrast, subcutaneous injection in the rat results in a low tumour incidence with a relatively long latent period.

It may well be that due to the insolubility and unreactivity of TCQ, percutaneous application represents a more favourable route for the presentation of the carcinogen to tissue. This is supported by the finding that subcutaneously injected TCQ becomes localised and encysted and little appears to be metabolised. In this connection, it is interesting that the induction of subcutaneous tumours with TCQ closely resembles the effects observed by Oppenheimer *et al* (1955) on tumour induction with plastics, since many of these substances are also chemically inert. Recently Oppenheimer *et al.* (1958) have shown that tumours arise from cells lining connective tissue pockets surrounding subcutaneously embedded plastics. Although the initial changes were brought about by the plastic films, the importance of the tissue pockets in the later stages of tumour induction was apparent since, after a certain time, it was possible to remove the embedded plastic without influencing tumour formation. These observations are of great interest since similar histological changes were observed during the induction of subcutaneous tumours with TCQ and it was found that all of the tumours arose in close association with TCQ cysts. Thus it would appear that, by virtue of its chemical inertness, the carcinogenic activity of TCQ administered subcutaneously in the rat is similar to that of plastics, although the primary mechanism of carcinogenesis may be quite different. The fact that TCQ possesses a much greater carcinogenic activity when applied to mouse skin raises the question as to whether polymeric substances may also show greater activity when applied in the same way and experiments to investigate this problem are now in progress.

The activity of TCQ on mouse skin is of about the same order as that of 1:2:5:6-dibenzanthracene (Hartwell, 1951) whose carcinogenic properties together with those of other polycyclic hydrocarbons and alkylated benzacridines have been interpreted in terms of electron densities at the K and L regions (Pullman and

Pullman, 1955 ; Lacassagne, Buu Hoï, Daudel and Zajdela, 1956). High activity is evident in certain members of these series having K regions of appropriate electron density and in which the L regions are also blocked. In inactive members the K regions are either absent or have electron densities of inappropriate values. Moreover, the introduction of blocking groups into the K regions of 1:2:5:6-dibenzanthracene suppresses its carcinogenic activity (Oliverio and Heidelberger, 1958). TCQ has no region which is logically identifiable as an L region or an unsubstituted K region. It therefore follows that the interpretation of its carcinogenic activity in terms of the union of a K region with an electrophilic cell-receptor (Pullman and Pullman, 1955) would appear inapplicable. In this connection it is of interest that preliminary studies on the metabolism of TCQ on mouse skin have failed to reveal the occurrence of protein-bound metabolites.

SUMMARY

1. The carcinogenic action of tricycloquinazoline has been investigated by—
   (*a*) subcutaneous injection in rats,
   (*b*) skin painting in mice.
2. In rats the tumour incidence is low (27 per cent) and the induction period long (16–24 months).
3. In mice the tumour incidence is high (72 per cent) and the induction period shorter (110–280 days).
4. Following subcutaneous injection the TCQ remained localised in a cystic space and tumours arose from its fibrous wall.
5. This lesion was in every way similar to that observed following the embedding of certain plastic substances.
6. The mode of action of TCQ in relation to other known carcinogens is discussed.

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MAMMARY CARCINOMA INCIDENCE IN C_3Hf VIRGIN FEMALE MICE AFTER OVARIECTOMY AND SUBSTITUTED OESTRONE

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WHEN 5 μg. of oestrone alone or combined with 50 μg. of progesterone were substituted weekly for 60 weeks after bilateral ovariectomy in RIIIf strain mice in which no evidence of milk factor has been found through 49 generations after cross-suckling, mammary carcinoma incidence was raised from none in intact virgin females to 1 in 39. In ovariectomised breeders the spontaneous incidence of 1 in 40 in intact females was maintained at about the same level, 1 in 31, by substitution of 5 μg. oestrone weekly after breeding. 50 μg. weekly failed to induce any carcinoma in 37 ovariectomised breeders. Owing to the low spontaneous incidence in RIIIf mice and therefore the large number of females that would be required, it was not feasible to try to determine whether or not bilateral ovariectomy after breeding would prevent the appearance of mammary carcinoma in later age (Pullinger, 1955, 1957). Other problems that could not be pursued for the same reason and also on account of a large increase in incidence of lymphoblastoma, concerned the variety in kind and quantity of other natural hormones and their metabolites which might be substituted. Would substitutions of oestradiol; oestriol or other biologically active metabolites or mixtures of these with oestrone give similar results? Is there a quantitative relationship between naturally occurring ovarian hormones and mammary carcinoma incidence in mice without milk factor? or is there a maximum incidence for each agent free strain, which can be reached but not exceeded whatever the dose of natural oestrogen whether endogenous or applied? Thanks to the generous gift to this hospital by Dr. W. E. Heston of 2 litters of C_3Hf/He mice it has been possible to continue this enquiry using another strain. Whereas the highest spontaneous incidence of mammary carcinoma seen in RIIIf breeding females was between 2 and 3 per cent after 1 year of age having borne at least 3 and up to 16 litters, the spontaneous incidence of C_3Hf/He breeders lacking evidence of milk factor was originally 38 per cent. At the time the 2 litters in the F 24 and 25 generations were received in 1954 the incidence had recently fallen. It has in the interim been found to have an overall value from F 6 to F 25 of 22 per cent (Heston, 1958). Particulars of origin, of cross-suckling after delivery by Caesarian section, of the subsequent examination of characters and of tests for evidence of milk factor have been recorded for this strain by Heston and his colleagues (Heston *et al.*, 1950; Heston and Deringer, 1952; Heston, 1953; Heston, Deringer, and Dunn, 1956; Heston, 1958). A comparison of characters of the progeny born since arrival in these laboratories is in progress. No evidence of infection with milk factor has been found and the overall incidence of mammary carcinoma in breeders is 24 per cent.

The disadvantage of using mice derived from an original C_3H stock in experiments entailing bilateral gonadectomy and the substitution of known amounts of ovarian hormones is the post-operative faculty of the adrenal cortex of this strain in common with several others (but not the RIII) to undergo morphological changes leading to hyperplasia, adenomatous and malignant growths (Woolley and Little, 1945*a*, *b* ; Smith, 1948 ; Frantz and Kirschbaum, 1949), with biological evidence of secretion of oestrogen. In C_3H mice ovariectomised at weaning age, Frantz and Kirschbaum (1949) found biological evidence of secretion of both oestrogen and androgen, sometimes from the same tumour. In ovariectomised NH mice bearing adrenal tumours Dorfman and Gardner (1944) found 4 times the normal amount of excreted oestrogen in extracts of urine and faeces. These morphological, neoplastic and biochemical changes were successfully counteracted in castrated Ce males by implanting under the skin 4·8 mg. pellets containing 25 per cent stilboestrol (Woolley and Little, 1946). Neither nodular adrenal hyperplasia nor tumours arose but tumours developed in pituitary glands. In the expectation that the amount of oestrone used in the present experiments would act similarly in preventing adrenal changes, substitution was attempted in C_3Hf mice ovariectomised at 8 to 10 weeks of age. In the event 5 μg. oestrone in actone applied weekly for 60 weeks to the skin did not prevent the development of foci of types A and B cells (described by Woolley and Little, 1945*a*) though no cortical tumours arose under these conditions and none in the pituitary gland. However, since B type cells appeared, the possibility existed that endogenous oestrogen might be secreted from them and become added to the measured amount given thus invalidating quantitative results. Evidence of secretion of sufficient endogenous oestrogen to cause vaginal cornification was found after ovariectomy alone at one time or another during the experimental period but evidence of physiological stimulation of mammary epithelium in the absence of added oestrone was definite in 2 only out of 28 mice neither of which developed mammary carcinoma. Both these mice had developed adrenal cortical carcinoma. It appeared therefore that the contribution of endogenous oestrogen from B cells of the adrenal or other possible sources was probably insufficient to affect results dependent upon the stimulation of mammary epithelium even though actual cortical tumours had developed. In preliminary substitution tests 1 mammary carcinoma arose in 68 ovariectomised females 34 of which had received weekly 5 μg. oestrone only and 34 had 5 μg. oestrone and 50 μg. progesterone for 60 weeks. The spontaneous incidence in virgin females was less than 2 per cent and average survival ages were 28 months for experimental and 27 months for intact females.

METHODS AND MATERIALS

After 4 generations, breeding was continued from one only of the 2 C_3Hf litters. Progeny not required for breeding were used in experiments. All animals set aside for experiments were examined weekly for tumours or other lesions. They were let live as long as possible and were killed only after tumours had been found or when they were unable to feed. After post-mortem examination nipple regions were fixed in Bouin's solution, bulk-stained with dilute haematoxylin, dehydrated, cleared and examined with a dissecting microscope magnifying 7 and 14 times. All other tissues were fixed as a routine in Bouin's solution or, for special stains, in Helly, Zenker or formol saline.

Four groups of mice were observed. The first of 118 virgin females was kept intact and was segregated for life after weaning. The females of the other 3 groups had bilateral ovariectomies, including a piece of uterine horn, done when they were 8 to 10 weeks of age. Thereafter one group of 40 had no further treatment; another of 39 was treated with 5 μg. oestrone once weekly and 50 μg. progesterone once weekly at 4 day intervals for 60 weeks. The oestrone and progesterone were applied in acetone solution to the whole length of the backs after clipping the hair by hand. Only those females which lived to 1 year or over were included in the Tables and comparative results. In all groups the numbers recorded for tumour incidence are slightly greater than those in which microscopic examinations of adrenals or nipple areas were made because some few were unsuitable on account of autolysis. When required tumours were grafted into females and males of the same strain or into F 1 hybrids.

RESULTS

Intact virgin females

One hundred and fifteen survived to 12 months of age or over and reached an average of 27 months. Two developed mammary carcinoma at 30 months. One of these tumours was a papillary cystadenoma with large amounts of precipitable secretion in many cysts (Type B ; Dunn, 1953) and was associated with an ovarian tumour. The second was an adenocarcinoma (Type A ; Dunn, 1953) without associated ovarian tumour. It was grafted and grew in all of 3 females and 5 males. All 10 nipple regions of 93 of this group were examined for development of hyperplastic nodules (adenomas). All had well developed duct systems and varied from complete involution to lobular alveolar differentiation equivalent to 9 days' pregnancy as judged by comparison with the silhouette method of Cole (1933). Hyperplastic nodules were found in old females of this strain by Jones (1951) and were seen in 27 mammae of the 93 examined in this group. The nodules could not be counted because not all mammae were involuted. The greatest number appeared to be 46.

Adrenal cortices of the 2 tumour-bearing mice contained abundant A cells. There was no nodular hyperplasia and a few foci only of B cells in each adrenal. The cytoplasm of cells of the zone fasciculata which normally appears vacuolated in paraffin sections owing to solution of lipoids in the process of embedding contained instead uniformly precipitable eosinophilic material with no or few vacuoles. The cells were larger than normal and were a conspicuous feature in all but 1 out of 66 pairs of adrenals examined in this group. As they became enlarged in intact females or sub-divided into groups by encroachment of clear B cells in those other groups which were ovariectomised, their original columnar arrangement was lost. They appear to correspond with eosinophilic cortical cells situated between Type A and degenerating X-zone referred to by Woolley and Little (1945*a*) who attributed the alteration in appearance to a metabolic disturbance, and by Smith (1948) as swollen fasciculata cells. In the present material no fine cytoplasmic granules could be seen with Bouin, Helly, Zenker or formol fixation. With increase in age scattered and irregularly shaped and sized yellow granules were found. As compared with the pigment of "brown degeneration" (chromolipoid, ceroid) which was also present in older animals, these granules were sparse and only the larger among them were acid fast by Ziehl-Nielsen's method. Nuclei were lepto-

chromatic with one or more prominent nucleoli and were situated eccentrically. Two or more nuclei were sometimes seen but no mitoses, yet a few extracapsular adenomas of these cells were found There was a superficial resemblance to lutea cells of the ovary in pregnancy but centrosomes could not be found. These large eosinophilic cells differed completely from the lutein-like cells of atretic follicles described by Fekete, Woolley aud Little (1941). In another series of observations (unpublished) the change from typical lipoid containing to eosinophilic cytoplasm was first found in a minority of cells at 11 months and in the majority at 16 months in intact virgin females. In 65 out of 66 pairs of adrenals in the present series subcapsular A and eosinophilic fasciculata cells were found in all. B cells were hyperplastic in 3 and a few groups were seen in 39 animals. A pair of adrenals in 1 animal only aged 23 months was normal and still contained X-zones. Discrete adenomas of eosinophilic cells about 0.5 mm. in diameter were found outside the adrenal capsule in 6 animals. Others may have been missed.

There is a morphological similarity between these mouse fasciculata cells and human compact adrenal cells found after stress or ACTH stimulation (Symington and Davidson, 1956; Symington, Duguid and Davidson, 1956; Symington *et al.*, 1958) Though the cytoplasm of the mouse cells appears to lack fine granules, histochemical comparisons with human compact cells are being carried out.

TABLE I.—*Tumour incidence in Intact, Ovariectomised and Treated C_3Hf Virgin Female Mice Surviving for 12 Months or More*

	Number of mice	Average age in months	Mammary carcinoma (%)	Hepatoma (%)	Adrenal carcinoma (%)	Epithelioma (%)	Sarcoma (%)	Ovarian tumours (%)	Lung adenoma (%)
Intact	115	27	2 (1·7)	27 (23·4)	0	1 (0·86)	13 (11·3)	12 (10·4)	14 (12·1)
Ovariectomy only . .	30	28	0	7 (23·3)	2 (6·6)	0	2 (6·6)	—	2 (6·6)
Ovariectomy and 5 μg. oestrone weekly	34	27	1 (2·9)	5 (14·7)	0	1 (2·9)	3 (8·8)	—	1 (2·9)
Ovariectomy and 5 μg. oestrone; 50 μg. progesterone weekly	34	28	0	5 (14·7)	0	2 (5·8)	1 (2·9)	—	3 (8·8)

— = ovaries excised. Lymphoblastoma and leukaemia are excluded.

Tumours with the exception of those of the lymphoma-leukaemia group, small haemangioomas and mammary adenomas (which are recorded in the text) will be found in Table I. The lymphoblastoma group was excluded because blood counts were not done and because much evidence has been obtained in the course of this work of the existence of very numerous nodules and small foci of lymphoid cells in lungs, subcutaneous tissue, along branches of mammary ducts, in the abdominal cavity and in kidneys and perirenal tissue. All or none of these would have to be included and the search for them could not be complete. The 1 epithelioma arose in the skin. Ovarian tumours include sarcoma of this organ. Among sarcomas 2 were osteogenic. The pituitary gland was examined macroscopically and with the dissecting microscope in the 2 mice with mammary tumours and in many others which were freshly killed or in a good state of preservation. No tumours or enlargements have been found.

Bilateral ovariectomy alone

After ovariectomy alone, 30 out of 40 mice survived for 1 year or more, reaching an average age of 28 months. All were examined when dead to ascertain that no ovaries or fragments remained. Tumour incidence with exceptions previously named are listed in Table 1. No tumours arose before 1 year of age. Of the 30 survivors, all but 2 were in sufficiently good condition for microscopic examination of nipple regions and adrenal glands. Two only developed adrenal cortical carcinoma. Tests for vaginal cornification were done at intervals and at one time or another, and irregularly, squamous cells were found alone in all of this group. There was little evidence of stimulation of nipple regions except in the 2 animals with adrenal carcinoma. Scoring of evidence of stimulation was done in comparison with mammary glands at puberty, the age, or just over it, at which ovariectomies were done. At puberty, main ducts have developed but do not as a rule extend to the limits of the fat pads. Any doubtful extension short of these limits was given a $\pm$ score. When the limits of the fat pads were reached a $+$ was scored and $++$ when, in addition to outgrowth, there had been proliferation and branching of main ducts.

Both adrenal cortical carcinomas which developed were composed mainly of clear cells resembling type B. One was grafted and after 7 months 2 grafts had grown, one in an intact male and 1 in an ovariectomised female. Type B cells were found in all and were hyperplastic in 16 of the remaining 26 mice examined, or, including the mice with tumours, in 64 per cent after ovariectomy alone. Medullary cysts were seen in 7 adrenals and hyperplasia in one.

Bilateral ovariectomy and substituted oestrone

After ovariectomy and substitution of 5 μg. oestrone weekly for 60 weeks, 34 out of 39 mice survived for 1 year or over and reached an average age of 27 months. No adrenal carcinoma developed. One mammary carcinoma occurred in a mouse aged 29 months. Both adrenal glands of this animal contained some A, groups of eosinophilic fasciculata and hyperplastic B cells and pigmented cells. Definite duct growth and proliferation had occurred in the mammary gland and there was 1 adenoma. In nipple regions of the remainder of the group there was good evidence of stimulation in 2 and adenoma in 5 mice. The one carcinoma was an adenoacanthoma and it grew progressively from the time it was observed and in all 3 females and 10 males into which it was grafted.

Types A, B and eosinophilic zona fasciculata cells were found in the adrenal glands of all 31 mice examined indicating that the substituted oestrogen was either insufficient in quantity or kind or was applied for too short a time to prevent these changes. Type B cells were hyperplastic in 12 or 38·7 per cent compared with 4·6 per cent in intact females of nearly the same average age. One microscopic medullary tumour and 1 cyst but no cortical tumours were found. Other tumours are recorded in Table I. One sarcoma arose in the stump of a uterine horn (no ligatures were used). The epithelioma arose from skin. One sarcoma arose in a nipple region. Its nature was doubtful but it was finally classed as a sarcoma. rather than carcinosarcoma.

Bilateral ovariectomy and substitution of oestrone and progesterone

Thirty-four out of 39 mice survived to an average age of 27 months. Adrenal and mammary glands of 28 were examined at an average age of 28 months. No

adrenal carcinoma or mammary adenocarcinoma developed. A larger number, 13 as compared with 2 each in the other groups, showed considerable duct outgrowth and branching. The number with lobular-alveolar development was 12 compared with 2 and 3 in the other groups. Nipple regions of 1 resembled a gland at about 6 days' pregnancy. Nodules were found in 8.

Types A, B and eosinophilic zona fasciculata cells were found in all adrenals. There was hyperplasia of B cells in 9 or 32 per cent. The addition of 50 μg. of progesterone to the 5 μg. oestrone given in the previous group did not affect these changes. Other tumours are recorded in Table I. One epithelioma arose in skin and 1 in a clitoral gland. None occurred before 1 year of age. One medullary cyst, no medullary proliferation and one cortex composed of epithelial tubular structures surrounding a cyst were found.

COMMENT

When bilateral ovariectomy was done on C_3Hf females at 8 to 10 weeks of age the subsequent adrenal cortical changes were accompanied by enough oestrogen secretion to cause cornification of the vagina but insufficient to influence mammary epithelium physiologically unless cortical carcinoma developed. Even then, in these agent free females, no mammary carcinoma developed. At an earlier age this might not have happened for adrenal cortical changes of greater degree occurred in C_3H females after ovariectomy at 1 month of age (Smith, 1948). With the appearance of compact or luteal-like cells in the zona fasciculata, the possibility of secretion of progesterone had to be considered but of this no evidence was found. According to results, (Table II), when a small amount of progesterone was added its synergistic action was apparent in greater duct growth and lobular-alveolar differentiation than when oestrone alone was applied.

TABLE II.—*Mammary Gland Stimulation in C_3Hf Female Mice After Ovariectomy at 8 to 10 Weeks of Age and Substitution of 2 Ovarian Hormones*

	Ovariectomy only	Ovariectomy and 5 μg. oestrone weekly	Ovariectomy and 5 μg. oestrone and 50 μg. progesterone weekly
Number of mice	28	31	28
Average age in months	28	28	27
Duct growth :			
None	22	15	4
±	4	5	11
+	0	9	0
++	2	2	13
Lobular-alveolar differentiation :			
None	25	29	16
+	3	2	9
++	0	0	3
Number of mice with adenoma	1	5	8
Total adenomas	1	8	18
Carcinomas	0	1	0

It appears that the C_3Hf strain may prove suitable for testing the effect on mammary carcinoma incidence of varied amounts and combinations of substituted natural hormones including mammogenic hormones of the pituitary. Substitution of natural oestrogens might also be used to determine the amount secreted by

finding how much is required to prevent adrenal cortical changes. Though experiments are incomplete, it can be said at the present time that 10 μg. oestrone weekly is tolerated by the majority of ovariectomised animals but 20 μg. is not. Both amounts are associated with formation of urinary calculi and their consequences.

SUMMARY

1. Virgin female progeny of the C_3Hf/He strain of mice without evidence of milk factor were examined to determine if the strain would be suitable for testing the effect on incidence of mammary carcinoma of varied amounts of substituted ovarian hormones and their metabolites after ovariectomy at 8 to 10 weeks of age.

2. No mammary carcinoma arose in 30 females ovariectomised without substitution of oestrone. Definite evidence of physiological stimulation of the mammary gland was seen in 2 only out of 28 examined, both of which had developed adrenal cortical carcinoma. After substitution of 5 μg. oestrone weekly for 60 weeks 1 mammary adenoacanthoma arose in 34 ovariectomised females and none in 34 when, in addition to 5 μg. oestrone, 50 μg. progesterone was added. The spontaneous incidence in intact virgin females was 2 in 115. No adrenal cortical tumours arose after substitution of the two hormones.

3. The contribution of endogenous oestrogen attributable to the adrenal cortex or other source appeared to be negligible in effect on mammary epithelium.

4. The incidence of other spontaneous tumours in intact females is recorded and their incidence under the conditions of the experiment.

5. Attention is drawn to the change from lipoid containing to compact appearance of the cells of the zona fasciculata with age or ovariectomy.

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THE INFLUENCE OF SEX AND CASTRATION ON THE INDUCTION OF SKIN TUMOURS IN MICE BY METHYLCHOLANTHRENE

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It is known that epidermal mitosis is stimulated by androgens (Montagna, Kenyon and Hamilton, 1949) and also by oestrogens (Bullough and van Oordt, 1950). These hormones may, therefore, be expected to promote the rate of skin tumour development by chemical carcinogens provided there is no antagonism between the hormones and the particular carcinogen concerned.

However, on the basis of studies of the effect of methylcholanthrene on mouse vagina and seminal vesicle, Jackson and Robson (1957) have suggested that this carcinogen may affect the metabolism of steroid hormones, possibly by a competitive antagonism. Hamer and Marchant (1957) found a sex difference in the amount of collagen in mouse skin. Methylcholanthrene painting made the collagen compositions of male and female mouse skins approach each other, and this would be compatible with a hypothesis of antagonism. Jull (1956) suggests that methylcholanthrene has a progesterone-mimetic effect on mouse breast tissue, but he indicates that oestrogen may be an essential synergist in the induction of breast tumours with this carcinogen.

At the time when oestrogens had been proved to be important aetiological factors in the experimental production of breast tumours, a few studies were made on the influence of these hormones on skin tumour induction by tar and various carcinogens. The results were somewhat conflicting and may have been influenced by the particular carcinogen used. Gilmour (1937) found the carcinogenic response of the skin to benzpyrene paintings was increased by treatment of both males and females with oestrone. More oestrogen-treated mice than controls developed tumours and they tended to appear earlier. Perry and Ginzton (1937) compared the development of skin tumours in intact and spayed female mice painted with 1 : 2 : 5 : 6-dibenzanthracene, with and without simultaneous oestrone treatment. They encountered a heavy mortality, but were of the opinion that the incidence of skin tumours was not affected by spaying or oestrone treatment. Paletta and Max (1942) studied the influence of oestradiol treatment of intact female mice on the induction of skin tumours with methylcholanthrene. They found that papillomas appeared at about the same time in oestrogen-treated and control mice, but the time required for transformation to malignant tumours was reduced significantly by 1·8 weeks.

Recently there have been indications that a different yield of skin tumours may be obtained in the two sexes after treatment with chemical carcinogens and promoting agents. Salaman and Roe (1956) found that treatment with 9 : 10-dimethyl-1 : 2-benzanthracene followed by croton oil resulted in a far greater number of papillomas in male mice than in females, though the incidence of

malignant tumours was about the same. Berenblum and Haran-Ghera (1957) found the yield of skin papillomas after oral administration of urethane and subsequent croton oil skin paintings was greater in females than in males.

It seemed, therefore, that a study of the induction of skin tumours by methylcholanthrene in intact and castrated mice of both sexes might reveal some differences.

MATERIALS AND METHODS

Ninety male and 90 female albino mice from a closed colony were used in this investigation. At the age of 2 months, half of the males were castrated and half of the females ovariectomised.

Carcinogen treatment of all mice was begun when they had reached the age of 4 months. They were painted once weekly for 12 weeks with a 0·3 per cent solution of 20-methylcholanthrene in acetone. The carcinogen solution was applied liberally with a pipette to the skin on the right half of the dorsal surface of the animals, about 1 c.c. (3 mg.) being given at each painting.

Mice were housed in zinc cages, 5 animals per cage, and fed on a rat cube diet (Heygate and Sons, known as the Thompson's formula) with water *ad lib*. Weekly inspections for tumours were made and these were charted. Tumours were judged clinically malignant when they underwent an abrupt change, invading adjacent deeper tissues, and were no longer merely superficial papillomatous growths. Animals were kept until they died or until their condition made it necessary to kill them. When there was any doubt whether a tumour was a squamous carcinoma or not, it was removed at autopsy for histological examination.

RESULTS

Survival

There were originally 45 mice in each of the four groups of animals used. Those dying tumourless before the end of carcinogen treatment (11 weeks) were discounted. The mean survival of the remainder was similar in all four groups, being 27 weeks in intact females, 28 weeks in spayed females and intact males, and 29 weeks in castrated males. The rate of death was also similar in the four groups (Fig. 1).

Tumour Production

Of the 44 intact and 45 spayed females at risk, all developed skin tumours. One intact and 3 spayed females developed breast tumours in addition. All the 41 intact males at risk developed skin tumours, but 2 of the 43 castrated males died tumourless after 18 and 29 weeks respectively.

Rate of Appearance of Tumours

One of the most marked differences between the groups was seen in the rate of appearance of tumours. In the intact males and females the first tumours appeared 6 weeks after the first treatment with methylcholanthrene and the numbers rose steadily. In the castrated animals of both sexes, very few animals developed tumours until the 14th week, when there was a sudden steep rise in tumour appearance. By the 16th week there was little difference between all four groups.

Frequently papillomas regressed or were scratched off by the mouse. Sometimes they grew again in the same place. In assessing tumour production it is the common practice to discount tumours which subsequently regress and do not reappear. When this was done, the data presented below were affected in various ways, which will be considered in the appropriate places.

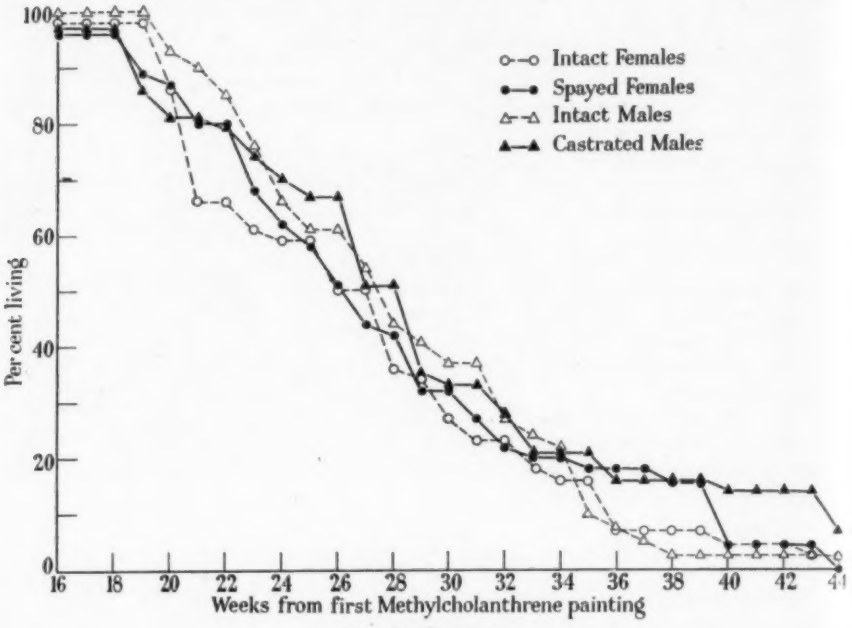

FIG. 1.—Survival.

Fig. 2 is a graphic representation of the appearance of tumours in the four groups of animals studied. When tumours which regressed were discounted there was very little effect on the shape of the histograms, the steady rise in tumour production in the intact animals being still sharply contrasted by the delay in castrates until the 14th week. It will be seen that discounting regressions had a relatively greater effect on intact females than on the other groups of animals.

The sudden steep rise in tumour production in the castrates of both sexes at 14 weeks can also be seen in Table I which shows the proportion of animals in each group bearing tumours from weeks 11 to 17.

When tumours which eventually regressed were discounted, the effect was to lower very slightly most of the figures in Table I. The figures for intact females were lowered rather more than those for the other groups, being reduced to numbers very similar to those for intact males.

A χ_2 test on the figures in Table I showed that at the end of carcinogen treatment (11 weeks) the tumour incidence was significantly higher in intact animals than in castrated ones of similar sex ($P < 0{\cdot}001$ in both cases). Although

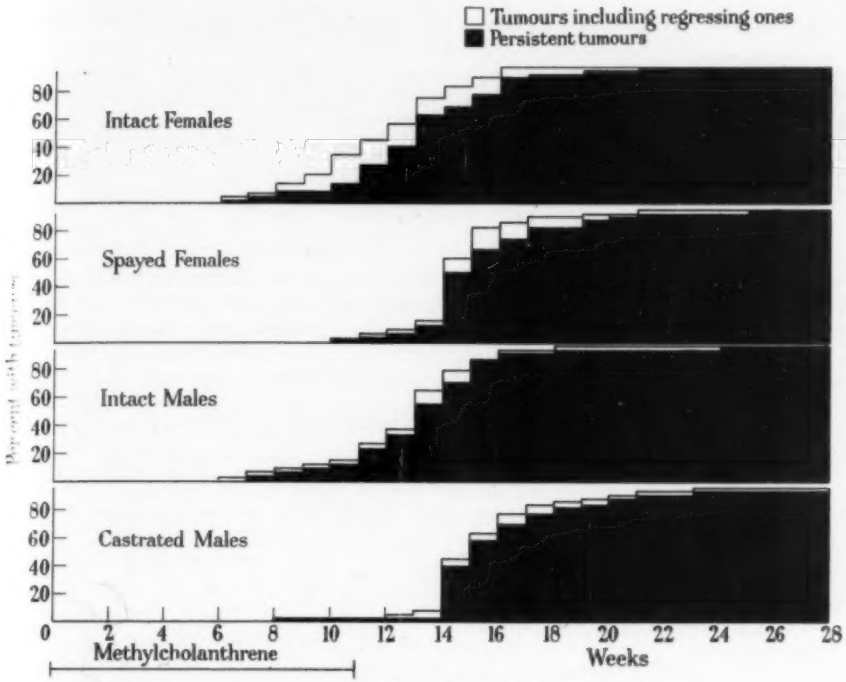

FIG. 2.—Onset of tumours.

TABLE I.—*Percentage of Animals With Tumours During Weeks* 11 *to* 17 *from commencement of methylcholanthrene painting*

	Week since commencement of painting						
	11	12	13	14	15	16	17
Intact females .	45	57	75	84	91	98	98
Spayed females .	7	9	15	60	82	87	91
Intact males . .	27	37	66	80	88	95	95
Castrated males .	2	5	7	44	63	77	84

the difference between intact males and intact females was just significant at the 11th week ($P = 0{\cdot}05$) it has already been explained that if tumours which later regressed were discounted there was no difference between these groups. The difference between intact and spayed females was no longer significant by the 15th week and that between intact and castrated males by the 16th week.

Table II also illustrates the rate at which tumours appeared. It shows the time by which a given proportion of animals in each group had developed tumours. If tumours which later regressed were discounted, there was very little effect on the figures given, except in the intact female group. Such differences are shown by the figures in brackets.

TABLE II.—*Weeks by which the Given Percentage of Mice had Developed Tumours (Alterations when Regressing Tumours Discounted).*

	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Intact females	8 (10)	9 (11)	10 (12)	11 (12)	12 (13)	12 (13)	13 (14)	14 (16)	15 (16)	31
Spayed females	13	13 (14)	14	14	14	15	15 (16)	15 (17)	19 (20)	31
Intact males	8 (9)	11	12	13	13	13 (14)	14	14 (15)	16	30
Castrated males	14	14	14	14	15	15 (16)	16	17 (18)	21	Not reached

The mean time of onset of tumours in each group of animals is given in Table III. Again a sharp difference between intact and castrated animals can be seen. If regressing tumours were discounted, the effect was to delay the mean time of onset in all groups by about one week.

TABLE III.—*Mean Time of Onset of Tumours*

	Onset of tumours (weeks)	Standard error	Onset of persisting tumours (weeks)
Intact females	12·33	±1·93	13·77
Spayed females	15·16	±2·32	16·14
Intact males	12·95	±2·07	13·54
Castrated males	15.45	±2·43	16·05

When figures in the first column of Table III were compared by the t test for significance, it was found that the intact animals of both sexes developed tumours faster than the castrated animals of like sex (P was just $< 0{\cdot}001$ in both cases).

Multiplicity and Regression of Tumours

Almost all mice developed more than one tumour, the maximum number produced by any mouse being 13. The number of tumours produced by mice in the four groups of animals is shown in Table IV.

TABLE IV.—*Percentage of Mice Producing the Numbers of Tumours Indicated or More*

	Total number of tumours developed per mouse					
	2+	4+	6+	8+	10+	12+
Intact females	100	93	72	44	26	14
Spayed females	98	82	47	31	16	5
Intact males	100	80	70	28	15	5
Castrated males	90	64	43	15	7	2

Frequently papillomas regressed or were scratched off by the mouse. Sometimes they grew again in the same place.

Table V shows the mean number of tumours produced per mouse, the mean number of regressing tumours per mouse and (by subtraction) the mean number of persistent tumours per mouse.

TABLE V.—*Mean Number of Tumours Per Mouse*

	Mean total number of tumours per mouse (including regressions)	Mean number of regressions per mouse	Mean number of persisting tumours per mouse (by subtraction)
Intact females	7·56±1·21	2·56±0·55	5·00
Spayed females	6·00±0·98	1·89±0·41	4·11
Intact males	6·50±1·10	1·65±0·40	4·85
Castrated males	5·07±0·88	1·67±0·40	3·40

A *t* test on the first column showed that the mean number of tumours produced by intact females was significantly higher than that produced by spayed females ($P < 0.01$). Similarly intact males produced significantly more than castrated males ($P < 0.02$). The difference between intact males and intact females was not significant (P about 0·08).

Although the mean number of regressions was greater in intact females than in the other three groups, this difference was not statistically significant ($P > 0.1$ for intact and spayed females).

The Onset of Malignancy

The clinical appearance of squamous carcinomata was spread over a great period of time, the earliest being found after only 8 weeks of treatment, the latest 47 weeks from the first treatment. The appearance of the first clinical signs of malignancy in the four groups of animals is represented graphically in Fig. 3.

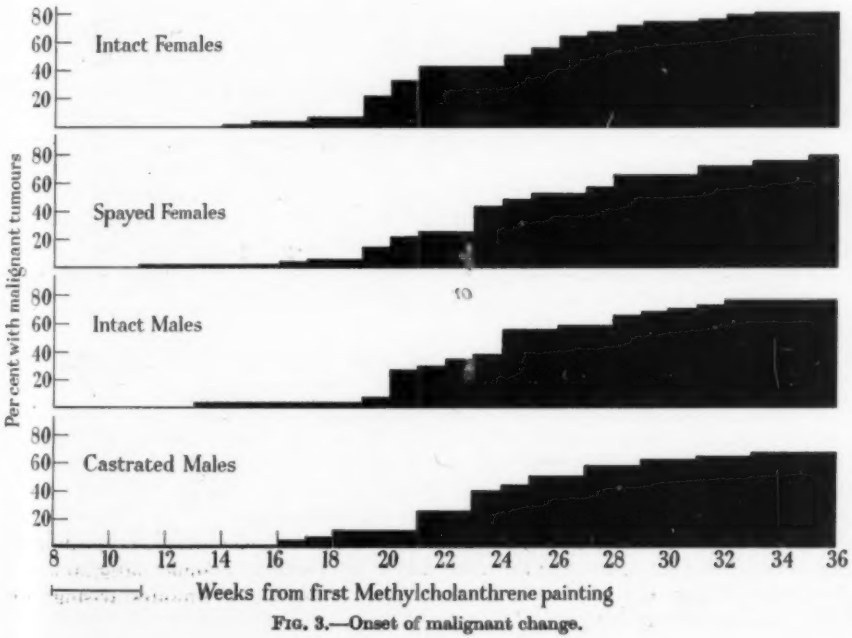

FIG. 3.—Onset of malignant change.

It is apparent that there was a fairly steady increase in appearance of malignant tumours in all groups of animals.

Table VI, which shows the rate at which the malignant change appeared, should be compared with Table II.

TABLE VI.—*Weeks by which the Given Percentage of Mice had Developed Clinically Malignant Skin Tumours*

	10%	20%	30%	40%	50%	60%	70%	80%
Intact females	19	19	20	21	24	26	27	32
Spayed females	19	21	23	23	25	28	31	35
Intact males	20	20	22	24	24	28	30	35
Castrated males	18	21	23	23	25	29	38	Not reached

The mean time of onset of malignancy is given in Table VII and was very similar in all the groups. It should be compared with Table III.

TABLE VII.—*Mean Time of Onset of Malignancy*

	Onset of malignancy (weeks)	Standard error
Intact females	22·8	±3·77
Spayed females	24·9	±4·10
Intact males	24·3	±4·13
Castrated males	25·2	±4·51

Thus, in spite of an earlier appearance of papillomas in intact animals of both sexes, as compared with castrates, malignant changes occurred in all groups at similar times.

It must be realised that multiplication of tumours was the rule in these mice and subsequent neoplastic change did not always occur first in the earliest papillomas which arose. In some mice more than one tumour became malignant, the maximum number being 3. A few tumours were judged malignant from their first appearance; other tumours which became malignant had appeared as much as 31 weeks earlier. It was considered that a comparison could be made between the rates at which individual tumours became malignant in all four groups. Table VIII shows the mean time taken by the individual tumours to become malignant, which was very similar in each of the four groups of animals.

TABLE VIII.—*Mean Time Taken by Individual Tumours to Become Malignant*

	Weeks to become malignant	Standard error
Intact females	7·9	±1·28
Spayed females	7·1	±1·30
Intact males	8·3	±1·63
Castrated males	7·7	±0·94

There was no correlation between the time of onset of the individual papillomas which became malignant and the time taken by them to become malignant, *i.e.* malignant change did not occur more quickly or slowly in tumours arising late than in those arising early.

DISCUSSION

The results given in Table III clearly show that, when intact mice of both sexes were compared with castrated animals of the same sex, the mean latent period before development of skin papillomas in response to methylcholanthrene painting was significantly shorter in intact animals. The number of tumours per mouse was also significantly greater in intact animals than in castrates (Table V).

It has been shown that mitosis in mouse epidermis is stimulated by both oestrogens and androgens (Montagna, Kenyon and Hamilton, 1949 ; Bullough and van Oordt, 1950). Since the induction of skin papillomas by methylcholanthrene appears to be enhanced rather than inhibited in intact animals compared with castrates, it may be that the sex hormones directly promote the effect of the carcinogen by virtue of this mitogenic action. It has indeed been shown that chemical induction of skin tumours in the rabbit is promoted by the increases in mitotic activity associated with zones of active hair growth (Whiteley and Ghadially, 1951).

The hypothesis of competitive antagonism between methylcholanthrene and the steroid hormones, advanced by Jackson and Robson (1957) appears to generalise too far in attempting to cover the tumour producing action of this substance. On their hypothesis " the steroid hormones should antagonise the carcinogenic action of methylcholanthrene on any organs, other than the secondary sex organs, which respond to stimulation by these hormones".

When we consider the onset of malignancy, it appears from Tables VI and VII that there were no significant differences between the various groups of animals. Table VIII also shows that the mean time taken by particular papillomas to become malignant was very similar in all groups. It must be realised that the assessment of whether a tumour was malignant or not was a rather more subjective matter than the assessment of the presence or absence of a papilloma. However, it was usually very obvious from one week to the next when a tumour had undergone a marked change resulting in invasion of surrounding tissues, and it is considered that the times given in the results are reasonably reliable.

It would seem from the results that in contrast to the onset of papillomas the onset of malignancy was not affected by gonadectomy. The two phenomena may well be influenced by different factors. This would lend further support to the idea illustrated by Foulds (1954) that progression of cells, by way of irreversible qualitative changes towards the aggressive characters of malignancy, takes place independently of the growth rate of the tumour.

SUMMARY

Twelve weekly skin paintings of intact and castrated mice of both sexes with 0·3 per cent methylcholanthrene in acetone resulted in a significantly earlier mean time of appearance of papillomas in intact animals than in castrates. The earliest tumours appeared in intact animals after 6 weeks and the numbers rose steadily until the 16th week, when 95 per cent or more of the animals had tumours. The tumour incidence before the 12th week was greater in intact females than in intact males, but when papillomas which subsequently regressed were discounted there was no significant difference between the sexes. In the castrated animals of both sexes there was a very significant delay in appearance of papillomas until the

14th week, when there was a sudden steep rise in tumour production so that by the 16th week the difference in tumour incidence between castrated and intact animals was no longer significant.

The mean number of tumours per mouse was greater in intact animals than in castrated animals of like sex.

There was no signficant difference in the time of onset of malignancy between any of the groups of animals studied.

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DEMONSTRATION AND SIGNIFICANCE OF INDEPENDENTLY GROWING MAMMARY CARCINOMAS WITHIN THE CARDIAC LUMINA IN MICE, WITH EPITHELIAL AND STROMAL DIFFERENTIATION

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In the course of the routine histological examination of the hearts of mice that died with mammary carcinoma, nodules of the tumor were found growing independently in the cardiac lumina, in a considerable number of cases. In the absence of any available earlier reference, it may be presumed that this is the first demonstration of the growth of carcinoma with formation of different histological patterns, consisting of both epithelial and stromal elements inside the cardiac lumina. The development of the stroma in a tumor growing in the medium of the circulating blood without direct contact of the host tissue contradicts the generally accepted theory that the stroma is supplied by the host tissue only and that for the maintenance and growth of the stroma, its direct continuity with the host tissue is essential.

The significance of this new finding with regard to the prognosis is also considered in this study.

MATERIALS AND METHODS

The material presented here is based on the autopsy study of C3H strain non-pregnant female mice, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The mice were allowed to die with mammary carcinoma, either spontaneous or transplanted. Tissues were fixed in 10 per cent neutral formalin and sectioned at 4 micron thickness. Hematoxylin and eosin stains were made routinely. Special stains for the detection of mucin, amyloid, iron, melanin, elastic tissue, reticulin, muscle, PAS positive material, etc., were used on some specimens. Single sections from the heart, other organs and the tumors were taken routinely. Serial sections were made of several hearts to verify the absence of attachment of the tumor embolus to the cardiac wall.

The analysis of metastatic spread, Table I, is based on the study of all autopsy cases in which the tumor, heart and lungs, together with other organs, were studied microscopically. This includes 21 mice with spontaneous and 684 with single transplanted carcinomas. These mice were used in our experimental chemotherapy program. Since no appreciable difference was noticed between treated and untreated groups, with respect to the histologic picture of the tumor,

TABLE I.—*Metastatic Spread of Mammary Carcinoma in Seven Hundred and Five C3H Female Mice*

No. of autopsies	Generation of tumor	No. of hearts showing tumor growth in cardiac cavities	No. of hearts showing coronary occlusion by tumor growth	No. of lungs with metastases	No. of animals having tumors in the cardiac cavity and no lung metastasis	No. of livers with metastases	No. of kidneys with metastases	No. of spleens with metastases
21	1st (i.e., spontaneous)	3 (14%)	–	6 (29%)	1 (5%)	–	–	–
370	2nd	16 (4%)	1	79 (21%)	2 (0·5%)	2	1	1
51	3rd	11 (22%)	1	21 (41%)	6 (12%)	1	–	–
129	4th	23 (18%)	2	40 (31%)	10 (8%	4	2	–
134	5th	15 (12%)	1	55 (41%)	3 (2%)	–	–	–
Total 705		68 (9·6%)	5 (0·7%)	201 (28·5%)	22 (3·1%)	7 (0·9%)	3 (0·4%)	1 (0·1%)

both of these groups were included in this study. Among the transplanted carcinoma group there were 370 tumors of second generation, 51 of third generation, 129 of fourth generation and 134 of fifth generation. None of the hearts were opened during the autopsy examination. The whole heart was fixed without knowing whether or not a tumor embolus was present within.

Sections of the rarely enlarged nodes show metastatic tumor in less than 1 per cent of the total autopsy cases.

OBSERVATIONS

The growth of the tumor inside the cardiac cavity, with the formation of various histologic patterns, is seen (Fig. 1 through 10). Epithelial and stromal characteristics of the adenocarcinoma of the breast are moderately well formed, as shown in solid alveolar (Fig. 1, 2), cystic papillary (Fig. 3, 4), acinous (Fig. 5), cystic (Fig. 6), and combined patterns (Fig. 9, 10). A tendency to capsule formation by the peripheral tumor cells is often seen (Fig. 1, 2, 6). Often the lining cell membrane appears as endothelial lining and mimics the appearance of tumor emboli lying in the lymphatic channels (Fig. 1, 2). Not infrequently formation of channels lined by a flattened tumor cell layer forming endothelium (Fig. 4) can be seen in these growing tumor emboli.

Cellular lysis is recognized in the tumor, resulting in the formation of cystic structures (Fig. 6) in a more or less solid tumor mass. Various sizes, ranging from small nodules to nodules large enough to fill right atrial (Fig. 9, 10) or right ventricular cavities (Fig. 7, 8) have been observed. Indentation and rupture of the cardiac wall, as a result of tumor growth inside the cardiac lumen is occasionally noticed (Fig. 8).

In sections of the heart, tumor nodules are occasionally noted in the lumen of the superior vena cava (Fig. 9) and in the pulmonary artery, while passing through the pulmonary valve (Fig. 8). Tumor nodules are seen only rarely in the cavity of the left atrium and left ventricle. The most common site is the right ventricular cavity ; next the right atrial cavity. The large size of the tumor emboli is a hindrance for its passage through the pulmonary valve and pulmonary artery. This

may partially explain the absence of metastases to the lung in several cases in which tumor emboli are seen in the cardiac cavity (Table I). Occasionally deposition of fibrin from the circulating blood is seen incorporated with the tumor (Fig. 1). On two occasions, the tumor nodule was seen to have been fixed to the lip of the cardiac valve cusp, but there was no evidence of continuity between valve tissue and stroma of tumor embolus.

In all the autopsy cases of mice that died with mammary carcinoma, either transplanted or spontaneous, microscopic study showed that the most frequent site of metastasis is the lung. We have observed the frequent occlusion of the pulmonary arteries and their branches by the tumor emboli with or without involvement of the lung parenchyma. Attainment of a large size by the tumor emboli in the pulmonary arteries, without invasion of the arterial wall for a considerable distance, is often noticed (Fig. 12, 13). Infarction of the lung is frequently seen associated with vascular obstruction due to tumor emboli (Fig. 12, 13). This has been one of the major complications leading to the death of the animal. In less than 1 per cent of the cases the coronary vessels were blocked by tumor emboli (Fig. 11, Table I), without any significant changes in the surrounding muscles because of the vascular obstruction.

The tendency for the mammary carcinoma in mice to maintain the form of a discrete nodule, even when it reaches an immense size, is shown in Fig. 14. This tendency is not only noticed in the primary tumor and the tumor nodules growing inside the heart but can be seen in the microscopic picture of the tumor while it is invading nearby tissue (Fig. 15, 16).

DISCUSSION

The presence of metastatic tumor nodules inside the heart, in various situations, shows their course along the route of venous blood circulation, i.e., from superior vena cava to right atrium, right ventricle, pulmonary artery and then lungs. For all practical purposes, it may be assumed that the metastatic spread to the heart is carried by blood vessels, since we have noticed many blood vascular channels showing tumor in their lumina and since the lymph node involvement by this tumor is very rare, as mentioned before and noticed by other workers. It occurred in less than 1 per cent of our mice. The frequent finding of occlusion of the large and main branches of the pulmonary arteries by tumor nodules is presumably the result of impaction of growing tumor emboli, which have already grown to be a large size in their sojourn in the cardiac cavity. Occlusion of the large pulmonary vessels leading to infarction of the lungs appears to be an immediate cause of death in a considerable number of animals.

It is also suggested from this study that in some cases the gain in size of the tumor nodules in the cardiac lumen stopped their further progression along the path of circulation. This probably explains the absence of metastases in the lungs in a few cases, while the cardiac cavity is filled with a large tumor nodule.

The marked tendency for this tumor to form a well-outlined nodule, as shown in Fig. 14, 15 and 16, probably plays an important role in the pattern of growth of tumor emboli inside the cardiac lumen and in the pulmonary arteries and may be related to the very infrequent metastasis to other organs.

Complete indifference of the mammary carcinoma to invasion of the heart muscle and endocardium is noteworthy. Indentation of the cardiac wall and,

finally, rupture because of the tumor growth still without invasion by the tumor, demonstrates the incompatibility of the cardiac muscle and endocardium with this tumor growth. Sugarbaker (1952) demonstrated by inoculating different tumors into the central arterial system of the rabbit that, in addition to establishment of its own metastatic pattern, a definite predilection exists peculiar to each tumor for the site of metastasis. His conclusion is that factors inherent in the type of tumor used and influences exerted by the organs of the host both contribute to produce these effects. Lucke, Breedis, Woo, Berwick and Nowell (1952) also show the differential growth of metastases in liver and lung in their experiments with Rabbit V2 carcinoma.

The growth and stromal differentiation of the tumor inside the cardiac cavity, independent of the host tissue, as shown in this study, is in contradiction to the generally accepted theory that the stroma is contributed by the host tissue only (Bashford, Murray and Cramer 1905*a*, 1905*b*; Haaland 1908). This theory emphasizes that, unlike the parenchyma, the stroma is devoid of the power of independent growth and proliferation and that the stroma of a tumor, either spontaneous or transplanted, introduced at inoculation undergoes degeneration and is replaced by supporting structures derived entirely from the tissues of the new host. It is important to recall that the detailed observations made by Loeb (1901) did not agree with this generally accepted theory that the stroma is derived from the host tissue only.

We have studied the various dynamic processes in the mechanism of formation of different tumor patterns and have observed the stromal differentiation by the neoplastic epithelial cells in mammary carcinoma of the mouse in both spontaneous and transplanted tumors (data to be published).

The growth of the tumor in the medium of the circulating blood, as presented here, probably represents an ideal condition of *in vivo* culture of the tumor for its epithelial and stromal differentiation. It is conceivable, as newer tissue inter- and intra-relationships are discovered, that continual or repeated supply of extracts or preparations from different tissues and organs could maintain the tumors under study more or less in their original structural and functional states entirely *in vitro*.

SUMMARY

Ten percent of seven hundred and five C3H strain female mice bearing either transplanted or spontaneous mammary carcinoma showed development of tumors with stromal and epithelial differentiation inside the cardiac lumen. The finding that these tumors had no direct connection with the host tissue, except the circulating blood, disputes the generally accepted theory that the stroma develops from the host tissue only.

The increase in size of the tumour growing inside the cardiac lumen has its implications for the impediment of cardiac function by obstruction, and also with regard to the pattern of metastatic spread, as shown and discussed in this study.

This investigation was supported by the Copher Research Fund.

Grateful acknowledgement is made to Mrs. Dixie McGregor; Miss Fern Probstmeyer and Miss Tutter Levinsen for technical assistance, Mr. Cramer Lewis,

Department of Illustration, Washington University School of Medicine, for photomicrographs, and Mrs. Rose Valle for secretarial help.

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EXPLANATION OF PLATES.

PLATE I

FIG. 1.—Multiple tumor nodules are lying inside the right ventricular cavity, Note the presence of delicate cell membranes (A) loosely encapsulating the tumor tissue, better shown in the following figure. Deposition of fibrin can be seen on the surface of two nodules. Tumor emboli growing inside the pulmonary arteries are shown in Fig. 13 in a section of the lung. 5th generation tumor. × 40.

FIG. 2.—This is a higher magnification of the preceding photomicrograph (A), showing the formation of the capsule by the peripheral tumor cells. 5th generation tumor. × 380.

FIG. 3.—Metastatic tumor forming a multiloculated cystic papillary pattern inside the right ventricular cavity of a mouse bearing mammary carcinoma. No connection of this tumor with the cardiac wall is seen in serial section ; the cardiac wall is free of tumor tissue. Note the delicate pattern of the tumor with well-differentiated stroma. A magnified view of the tumor tissue (A) is shown in the following figure. 2nd generation tumor. × 45.

FIG. 4.—This is a magnified view of a portion of the preceding photomicrograph (A). A narrow channel (pointed by an arrow) lined with endothelium-like flattened cells is shown here. 2nd generation tumor. × 340

PLATE II

FIG. 5.—Tumor emboli in the right ventricle forming an acinar pattern are exhibited here. No lung metastasis was seen in this animal. 2nd generation tumor. × 54.

FIG. 6.—Two isolated tumor nodules growing inside the right ventricular cavity are shown here. Note the formation of the capsule. Necrosis and dissolution of necrotic tumor tissue are seen in the formation of cystic cavities. No lung metastasis was seen in this animal. 4th generation tumor. × 38.

FIG. 7.—The right ventricular cavity is distended with a tumor growth. Prominent necrosis and hyaline degeneration can be seen in the tumor. No lung metastasis was seen in this case. Spontaneous tumor. × 22.

FIG. 8.—This photomicrograph demonstrates rupture of the right ventricular wall, due to the growth of the tumor inside the right ventricular cavity. Note the indentation marks over the inter-ventricular septum made by the growing tumor. A tumor nodule on its way to the pulmonary artery is shown on the left. The tumor nodule is seen lying against a pulmonary valve cusp. Lung metastasis was present in this case. 4th generation tumor. × 13.

PLATE III

FIG. 9.—The right atrial cavity is distended with the growing carcinoma, which shows combination tumor patterns. The two cusps of the right atrio-ventricular valve are shown below the main tumor growth. The atrial wall is thickened. A tumor embolus can be seen in the inter-atrial septum distending a coronary vessel. The mitral valve cusps can be seen in left center. A tumor nodule on the right upper corner distends the superior vena cava. Lung metastasis was present in this case. 3rd generation tumor. × 27.

FIG. 10.—The growth of a delicate metastatic tumor, showing a combination of different histological carcinomatous patterns can be seen in the right atrium, mostly surrounded by blood. Tricuspid valve cusps point downward. The left atrial cavity of this heart, which is not shown here, also contains a small tumor nodule. 2nd generation tumor. × 40.

FIG. 11.—In the inter-ventricular septum of a heart, a coronary vessel is filled with solid carcinoma cells. No other tumor metastasis was seen in the heart. Section of the lungs showed pulmonary metastasis. 5th generation tumor. × 180.

FIG. 12.—This photomicrograph shows a portion of a lung at its hilum. The tumor growing inside the pulmonary artery is seen here. The arterial wall in most part is not attached to the tumor, which has a rounded margin. At higher magnification, some of the peripheral tumor cells were seen to take the form of flattened endothelium. The rest of the lung shows hemorrhagic infarction. 2nd generation tumor. × 24.

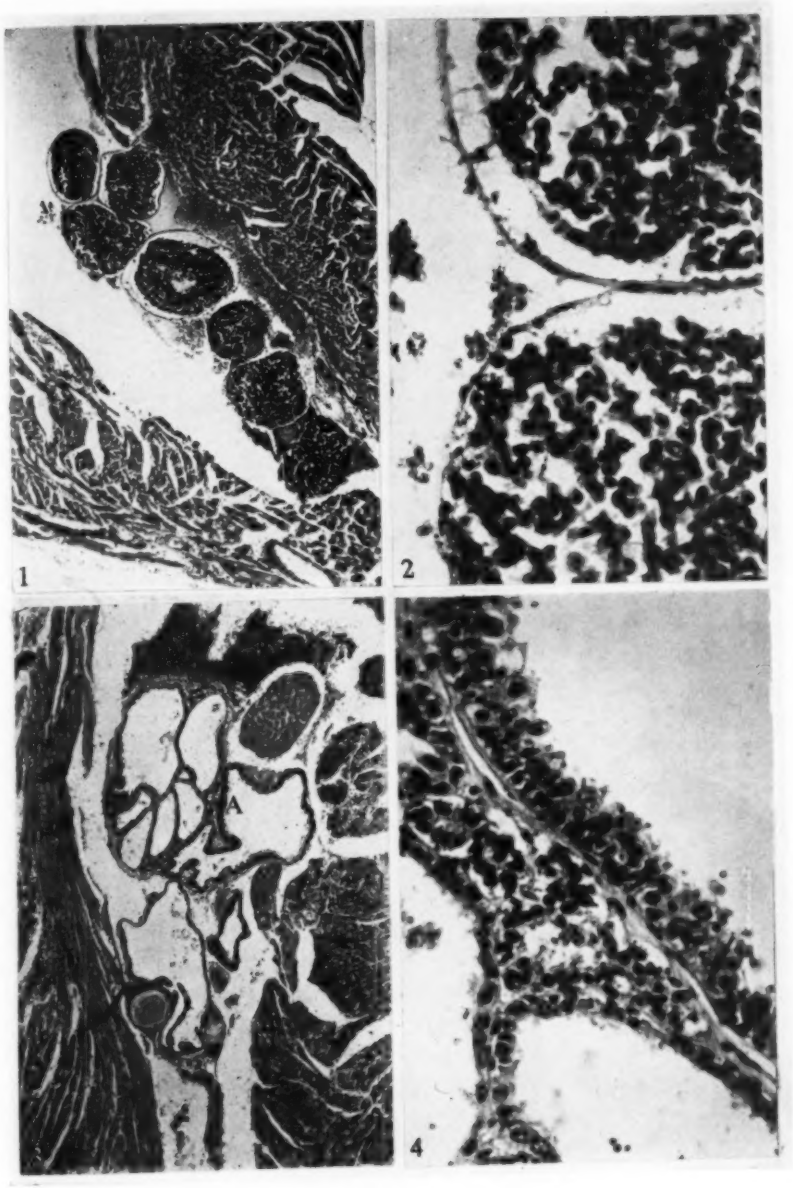
PLATE IV

FIG. 13.—This photomicrograph shows blockage of the pulmonary artery by the growth of the tumor embolus and infarction of the lung. Figure 1 is also from the same mouse. 5th generation tumor. × 33.

FIG. 14.—A C3H strain mouse with spontaneous breast carcinoma. Note the well-outlined tumor forming a large discrete nodule. Spontaneous tumor. × $\frac{1}{4}$.

FIG. 15.—This photomicrograph exhibits invasion of the chest wall muscle by the mammary carcinoma as a block—a frequent feature of this type growth. 1st generation tumor. × 70.

FIG. 16.—Invasion of the chest wall musculature by the tumor is shown here at a lower magnification. Note the pattern of invasion ; the invading tumor nodules have well-outlined boundaries. 4th generation tumor. × 26.



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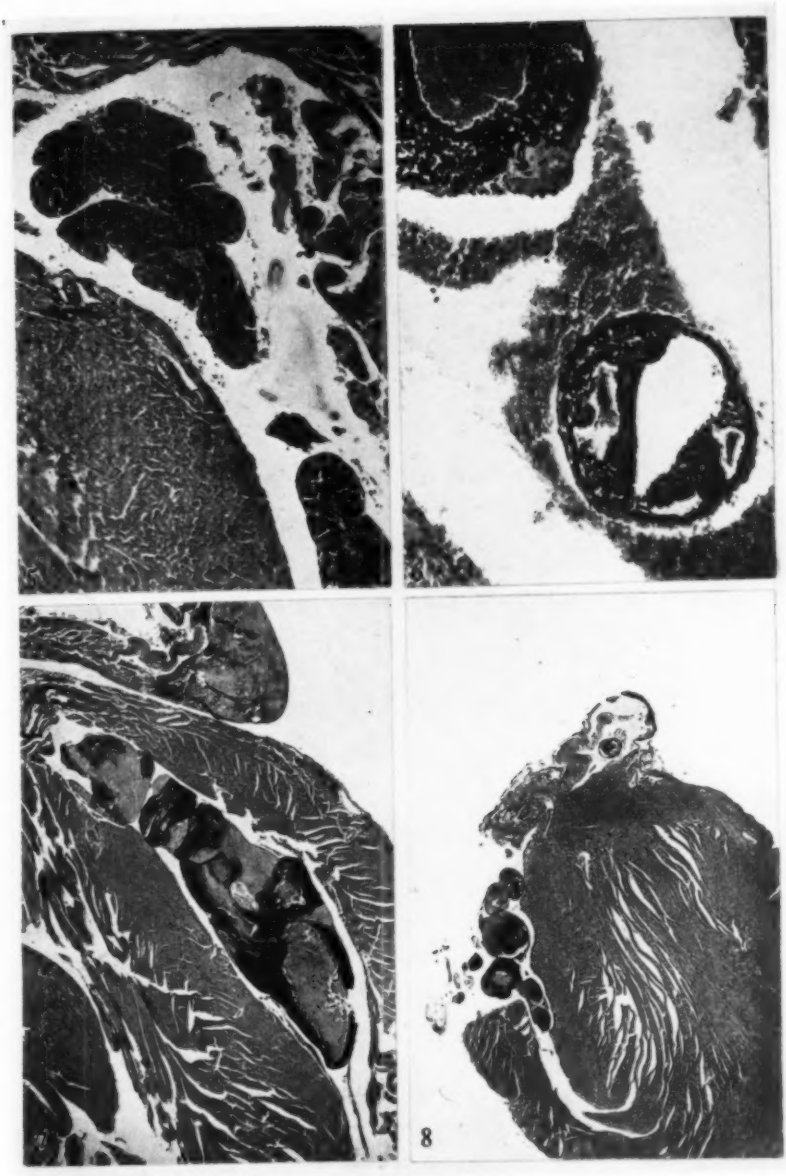

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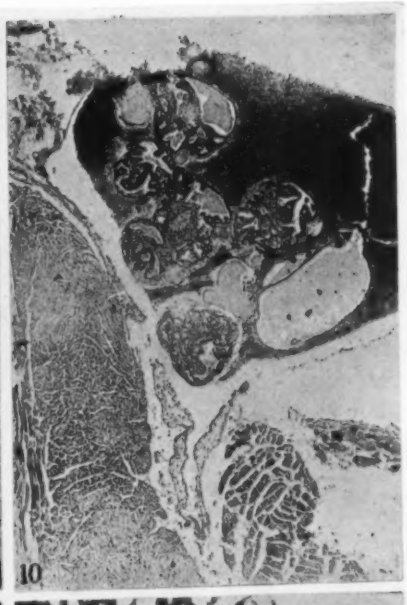

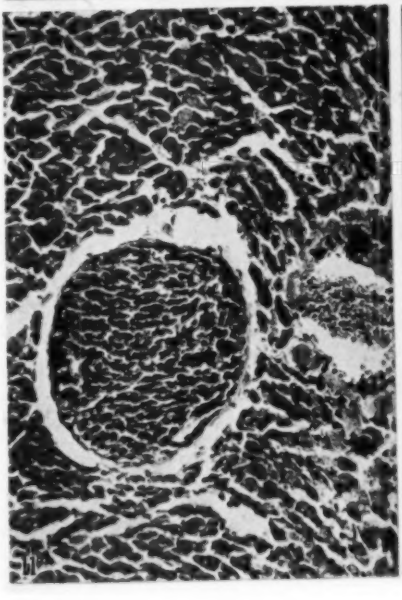

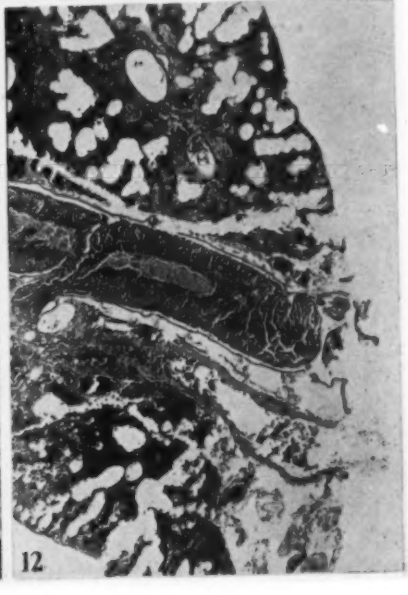

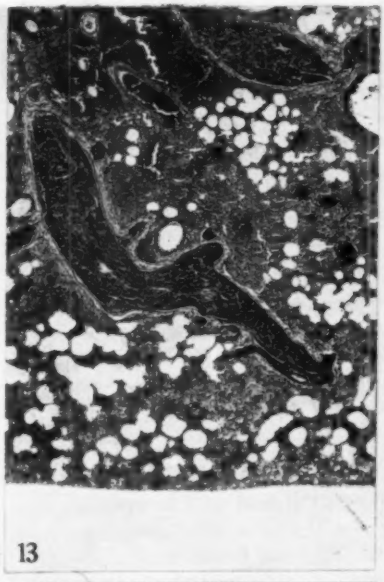

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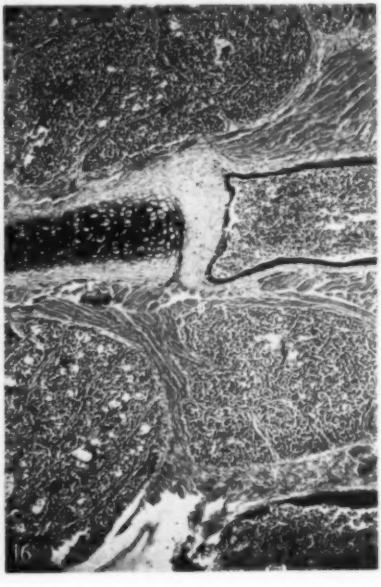

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AN INTESTINAL CARCINOMA IN MICE FOLLOWING INJECTION OF HERRING-SPERM DEOXYRIBONUCLEIC ACID

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As a preliminary to an attempt to incorporate in a living animal the deoxyribonucleic acid (D.N.A.) from a malignant tumour, it was thought advisable to perform a number of control experiments including one with a preparation of D.N.A. having no relation to any tumour, so as to exclude a non-specific effect. For this purpose a commercial product derived from herring-sperm was employed (Hewer and Meek, 1958). This was a depolymerised sample extracted by a hot alkaline method.

METHOD

A single litter of four female line-bred C_3H mice*, three weeks old at the start of the experiment, was used. Two of the mice were injected subcutaneously daily for five days with 0·1 ml. of 5 per cent D.N.A. solution. The other two were used as controls and received no injections at all.

RESULTS

On the 23rd day of the experiment one of those receiving injections was found dead and the other was moribund. The latter was killed immediately and selected tissues were fixed in Bouin's Fluid. The two other litter mates remained healthy and have developed since into normal adult mice subsequently killed at the age of one year. In both mice that had received injections there was a copious blood-stained peritoneal exudate and a firm pale grey ulcerating carcinoma, about 1 cm. in diameter, arising in the mucosa of the third part of the duodenum. The histological structure of these two tumours was the same, but since there had been some post-mortem autolysis of the mouse that was found dead a detailed study was made only of the other.

Both tumours had penetrated the gut wall and spread over the serous surface, and had also invaded the pancreas. There were some tumour cells free in the peritoneal fluid together with a few histiocytes and lymphocytes; in fact the tumour had already become established as an ascites-cell tumour, with more than 70 per cent tumour cells in the exudate.

MORBID ANATOMY

Since the lesions in the two mice were identical but the one found dead had suffered some post-mortem autolysis the following detailed description is based upon the findings in the other.

* The original breeding pairs used to establish this colony were kindly supplied by Dr. J. Craigie, Imperial Cancer Research Fund, Central Laboratories, Mill Hill, London.

The primary tumour arose in the mucosa of the third part part of the duodenum on the mesenteric side but had extended almost completely around the wall without, however, causing any obstruction. There was a small central area of ulceration of the mucosa and the main tumour measured approximately 8 mm. in diameter; it had invaded the mesentery and had extended into the body of the pancreas without causing any biliary obstruction. On section the tumour was rather friable, of uniform consistency with a few small yellowish necrotic foci, and involved the mucosa and the adjacent pancreatic tissue to such an extent that it was not possible in the gross to determine the exact site of its origin. Some loops of small and large intestine were loosely adherent to the outside of the tumour mass where some small nodules of tumour which had invaded the serous coat had given rise to the peritoneal spread. The mesenteric and para-aortic lymph nodes contained obvious metastases. (In the other mouse there was in addition a metastasis in one of the inguinal lymph nodes). These lymphatic metastases had penetrated the capsule of the lymph nodes and spread into the surrounding tissues to some extent. No metastases were found in the liver or in any extra-abdominal site.

Microscopically, the tumour proved to be a poorly differentiated adenocarcinoma arising from the deeper parts of the mucosal crypts. It was extremely cellular with finely granular cytoplasm and indistinct cell outlines. Most of the tumour was quite anaplastic but fortunately in some areas (Fig. 1) a transition was clearly perceptible between the normal duodenal mucosal crypts and those that had become carcinomatous. Mitoses were numerous and the cells varied considerably in size. The nuclei were vesicular with prominent nucleoli (Fig. 2). In the places where the pancreas was invaded sheets of tumour cells surrounded the pancreatic acini, the cells of which were mostly smaller than those of the tumour and generally distinguishable from them. In the lymph nodes the tumour cells gave no more than a suggestion of acinar arrangement.

Methanol-fixed smears of the ascites cells were stained by the May-Grünwald-Giemsa method and others fixed in buffered neutral formol-saline were stained with Oil Red O. The cells were mostly spherical and varied between 20 and 30 μ. in diameter. Fat droplets were present in the cytoplasm of almost all the cells and a very few showed cytoplasmic budding. The cytoplasm tended to be basophilic. Numerous bizarre nuclear forms were encountered including a few that were binucleate; in one of the latter (Fig. 3) there was a small nuclear protrusion present. Occasional giant cells were seen.

TRANSMISSION OF THE TUMOUR

Attempts were made to pass the solid tumour into other C_3H mice subcutaneously; these were successful in only three of six mice, and in these three subsequent passages could not be achieved. On the other hand the ascites fluid containing the ascites cell form, produced a vigorous growth of the tumour on

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EXPLANATION OF PLATE

FIG. 1.—Primary tumour showing transition between normal and neoplastic tissue. Haemotoxylin and eosin. × 195.

FIG. 2.—Primary tumour showing cellular detail. Haemotoxylin and eosin. × 390.

FIG. 3.—Ascites tumour cells including a giant form. May-Grünwald-Giemsa. × 650.

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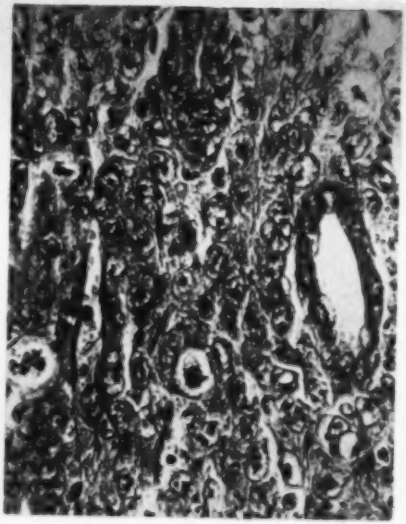

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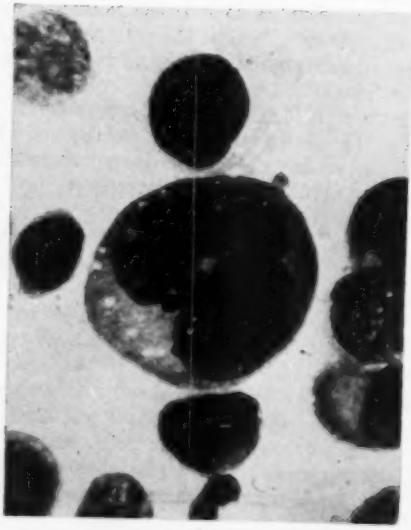

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intra-peritoneal injection with both C_3H and an inbred laboratory strain of albino mice, and has been maintained over the past year.

CYTOLOGICAL INVESTIGATIONS

Since the purpose of this experiment was to see whether the genetic structure of the cells could be altered by administration of D.N.A., studies were made of the chromosome and D.N.A. content of the tumour. This was more conveniently achieved with the ascites cells than with the solid tumour.

Chromosome counts were made on tumour cells arrested in metaphase by the administration of colchicine (6 μg. per 10 g. body weight) intraperitoneally to mice already bearing the ascites cell tumour. The cells were harvested at 10 hours after the administration of colchicine and were fixed immediately in aceto-orcein solution. Squash preparations were made and the chromosomes counted with the aid of a simple ocular grid. Most of the cells proved to be triploid, the count in individual cells varying between 56 and 63. In addition to this main group there was a small number of cells with a chromosome number lying between 120 and 130. Two cells were also found with a tetraploid figure of 80 and one cell with approximately 180 chromosomes.

D.N.A. estimations were carried out using a slight modification of the method and apparatus of Wolff (1957) to measure the intensity of Feulgen staining. A 108-watt tungsten ribbon filament lamp was used as the light source for projection of selected cells onto a screen fitted with a photo-electric cell behind a small aperture. Amongst the tumour cells there were lymphocytes present in the peritoneal exudate which could be distinguished from the tumour cells in Feulgen preparations. Since lymphocytes are normal somatic cells, and are therefore presumably diploid, measurement of their D.N.A. content would give normal values as a standard for comparison with the tumour. This amounts to a convenient built-in standard to minimise errors due to variations in staining intensity.

The results show (Fig. 4) that there is very little scatter of D.N.A. values in the normal lymphocyte nuclei whereas those of the tumour show in the main greatly increased values for D.N.A. content and a very much wider scatter, ranging from a few at normal level to a single reading of as much as about ten times the normal. It will be seen from Fig. 4 that the majority of the D.N.A. values lie in the range of twice to four times that of the normal cells.

The sources of error in this method for gauging D.N.A. content have been studied (Ris and Mirsky, 1949; Naora, 1955). It is recognised that there is difficulty in reproducing exactly the intensity of staining in any two preparations; this and other difficulties are increased by the lack of nuclear homogeneity. We were encouraged by the small degree of scatter in the values obtained for normal lymphocyte nuclei to feel that the use of these naturally occurring standards in our preparations did much to meet these objections.

DISCUSSION

Although we have as yet been unable to initiate any more tumours in this way our findings are of sufficient potential importance to warrant serious consideration. Firstly, we cannot find any report of the natural occurrence of an adenocarcinoma of the duodenum in C_3H mice; secondly, a triploid tumour is not

common and, thirdly, its appearance in both the mice injected with D.N.A. suggests interference with nucleic acid metabolism.

The original sample of D.N.A. used was small and no more of that batch was available. Another sample was used for later experiments and gave negative results. It is possible that these were not chemically identical and that the first contained some component lacking in the second. Hall and de Ropp (1955),

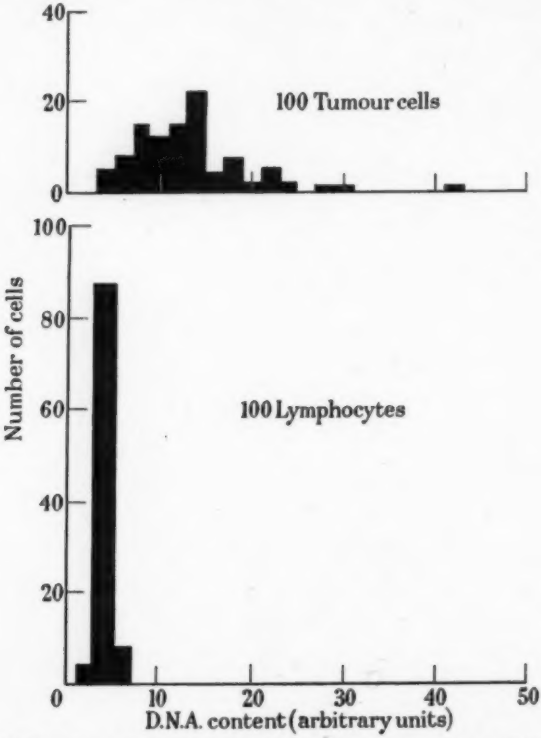

FIG. 4.—The D.N.A. values of 100 lymphocytes compared with those of 100 tumour cells.

showed that among the breakdown products of D.N.A. that may appear on storage or after autoclaving is kinetin (furfurylaminopurine) which, they concluded, might be formed by the interaction of adenine and 2-deoxy-D-ribose. It is possible that a product of this type had been formed in the sample of herring-sperm D.N.A. and become incorporated, on injection, in the D.N.A. of cells in the mucosa of the gut which were dividing very rapidly in these young mice.

SUMMARY

Duodenal adenocarcinoma appeared in two young mice given repeated injections of herring-sperm D.N.A. The tumours appeared identical. The histological

structure, including chromosome counts and estimation of D.N.A. content of nuclei, is described.

We have pleasure in acknowledging the full financial support given by the University of Bristol Cancer Research Fund.

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VEHICULAR EXHAUSTS: IDENTIFICATION OF FURTHER CARCINOGENS OF THE POLYCYCLIC AROMATIC HYDROCARBON CLASS

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THE recent epidemiological studies of Stocks (1957) in Great Britain and Hammond and Horn (1958) in the United States, while indicating a dominant role for cigarette smoking in most of today's lung cancer, have, in harmony with many previous surveys, shown the existence of an aetiologically significant urban factor. Haenszel and Shimkin (1956) in a statistical appraisal of surveys carried out in the United States, had already noted " the urban-rural discrepancy, in our opinion, represents a real finding and is a manifestation of multiple environmental factors in lung cancer ".

In a breakdown of the urban factor, Mills and Porter (1957) brought forward results which indicated the likelihood of a contributing role for motor exhaust fumes. These authors stated that driving mileages above 12,000 miles per year were significantly related to lung cancer incidence among urban men, except for those in the heavy smoking category. This result would seem to find support in the observations of Hueper (1957), that the lung cancer rate in Austria was about twice as high in communities located on main traffic arteries than in those situated remote from main highways, and Kretz (1953) who stated that, in Vienna, among the three most frequent sites of cancer, the lung takes first place for the following occupations: Traffic (9·7 per cent), Iron and Metal industry (8·7 per cent), Building (5·1 per cent), Hotel and Licensed Bar-keeping (3·6 per cent) and several branches of industry (17·2 per cent).

It is well known that during recent decades the rise in lung cancer frequency is paralleled by a similar rise in consumption of motor fuel. Benzene extracts of vehicular exhausts have been shown by Kotin, Falk and Thomas (1954, 1955) to produce skin cancers in mice.

As a means towards assessing the relative importance of vehicular exhaust-polluted atmospheres vis-a-vis cigarette smoking in the aetiology of lung cancer, at laboratory level, it would be desirable, firstly, to gauge the carcinogenic potency of the atmospheric extracts and cigarette smoke condensates in terms of, say, benzopyrene units of activity. Secondly, the necessity arises of comparing and contrasting the spectra of carcinogens in both forms of air pollution, the general and the individualised, with the view to furnishing a rational basis for the epidemiological results. The present paper, which records the identification of further carcinogens and other compounds in vehicular exhausts, contributes to the latter point and is an extension of previously reported work (Lyons and Johnston, 1957).

EXPERIMENTAL

Alternate adsorption chromatography on alumina and silica gel was carried out to purify fractions, isolated in the previous investigation from diesel and petrol engine exhaust soots. A number of compounds had since become available for reference. Identification of unknown compounds was made by comparing their ultra-violet absorption and fluorescence spectra (excitation radiation, 365 mμ.) with the spectra of reference compounds.

By these methods the following compounds have been newly identified in eluates succeeding 3,4-benzopyrene in petrol exhaust chromatograms: 3,4-benzofluoranthene, tetracene (naphthacene), pentaphene, 1,2,3,4-dibenzopyrene, 11,12-benzofluoranthene, 1,2,9,10-dibenzotetracene, 1,2,4,5-dibenzopyrene and 1,12,2,3-dibenzoperylene.

In the diesel exhausts, the following compounds were similarly identified: 3,4-benzofluoranthene, pentaphene, 1,2,3,4-dibenzopyrene, 11,12-benzofluoranthene, 1,2,9,10-dibenzotetracene.

Of the above listed compounds, the 3,4-benzofluoranthene, 1,2,3,4-dibenzopyrene and 1,2,4,5-dibenzopyrene are carcinogenic, while the 1,12,2,3-dibenzoperylene, it is believed, has not yet been tested. The structural formulae of these four compounds are shown in Fig. 1.

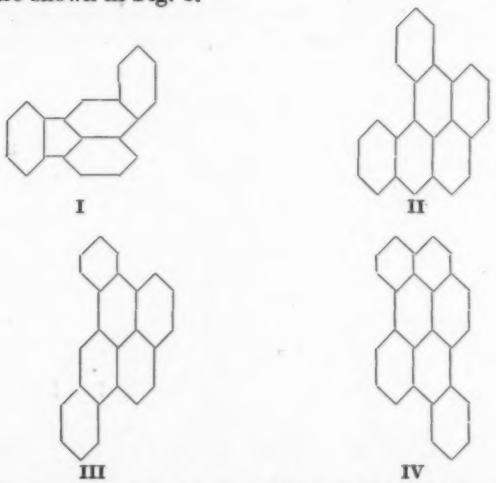

FIG. 1.—Structural formulae of compounds identified in vehicular exhausts.

I 3,4-Benzofluoranthene. II 1,2,3,4-Dibenzopyrene.
III 1,2,4,5-Dibenzopyrene. IV 1,12,2,3-Dibenzoperylene.

3,4-Benzofluoranthene has an intense blue fluorescence (the 11,12-derivative had an intense blue-violet fluorescence) and closely follows 3,4-benzopyrene on the chromatogram. The absorption spectrum of the compound from the petrol exhaust soot, which showed maxima at 368, 351, 303, 294, 275, and 256 mμ. in cyclohexane, is presented (Fig. 2). The fluorescence spectrum, which did not reveal any striking or discrete bands, consisted of a region of absorption commencing at 295 mμ. and having two peaks at 428 and 450 mμ.

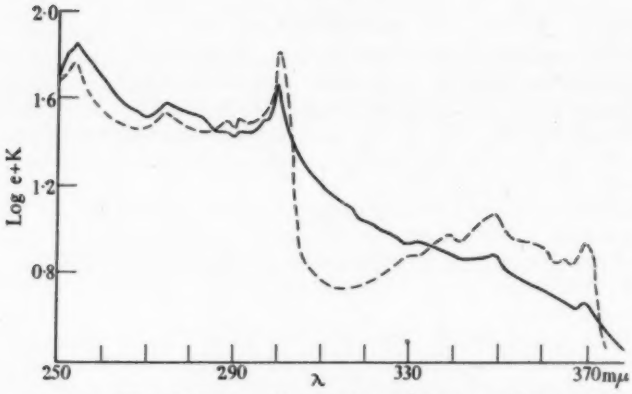

FIG. 2.—Ultraviolet absorption spectra: (solvent: cyclohexane).
——— 3,4-Benzofluoranthene from petrol exhaust soot.
- - - - Standard 3,4-Benzofluoranthene.

The absorption spectrum in benzene of 1,2,3,4-dibenzopyrene from petrol soot is shown in Fig. 3. The compound is associated with a green fluorescence.

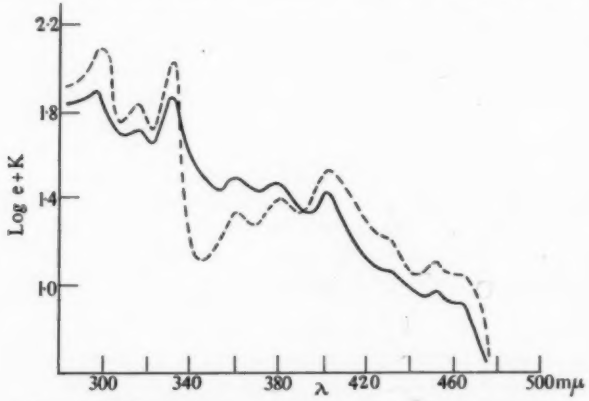

FIG. 3.—Ultraviolet absorption spectra: (solvent: benzene).
——— 1,2,3,4-Dibenzopyrene from petrol exhaust soot.
- - - - Standard 1,2,3,4-Dibenzopyrene.

The 1,2,4,5-dibenzopyrene eluates from petrol soot had a blue fluorescence. The absorption spectrum, in benzene (Fig. 4), gave peaks at 428, 416, 395, 378, 360, 327, 306, and 296 mμ., while fluorescence maxima at 416, and 440 mμ. were obtained.

Eluates containing the 1,12,2,3-dibenzoperylene had a blue-violet fluorescence. The absorption spectrum (Fig. 5) using benzene as solvent, showed maxima at 405, 391, 377, 357, 344, 309, and 297 mμ. A peak at 421·5 mμ. given by Clar

(1952) for this compound is now believed to be due to an impurity (Clar, personal communication). The fluorescence spectrum is distinctive and possesses a series

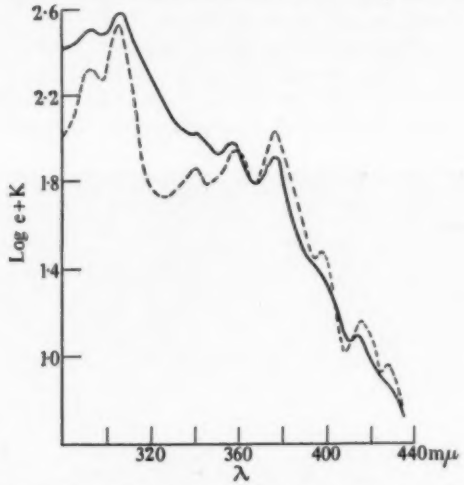

FIG. 4.—Ultraviolet absoprtion spectra : (solvent : benzene).
——— 1,2,4,5-Dibenzopyrene from petrol exhaust soot.
– – – – Standard 1,2,4,5-Dibenzopyrene.

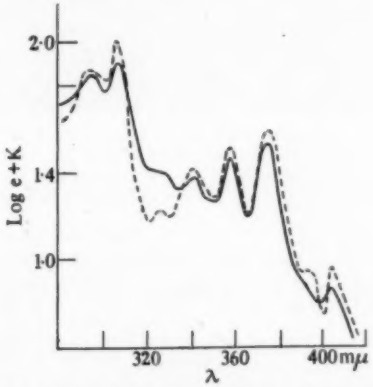

FIG. 5.—Ultraviolet absorption spectra : (solvent : benzene).
——— 1,12,2,3-Dibenzoperylene from petrol exhaust soot.
– – – – Standard 1,12,2,3-Dibenzoperylene.

of sharp bands at 404, 418, 428, 443 and 453 mμ. This spectrum was previously shown (Lyons and Johnston, 1957, Fluorescence Spectrum XVII) superimposed upon another band system which had maxima at 450, 467, and 478 mμ. Absorption maxima, in benzene, for the latter still unidentified compound, following chroma-

tographic separation from the dibenzoperylene were obtained at 422, 396, 369, 347, 328 and 304 mμ. The absorption spectrum of the incompletely purified compound was shown in Fig. 5 of the previous paper, while that of the then unidentified dibenzoperylene was shown in Fig. 4.

The fluorescence spectra of the benzofluoranthene, the two dibenzopyrenes and the dibenzoperylene are shown in Fig. 6. It has not yet been possible to estimate the 3,4-benzofluoranthene or the 1,12,2,3-dibenzoperylene. 1,2,3,4-Dibenzopyrene was found to occur at a concentration of approximately 22 parts

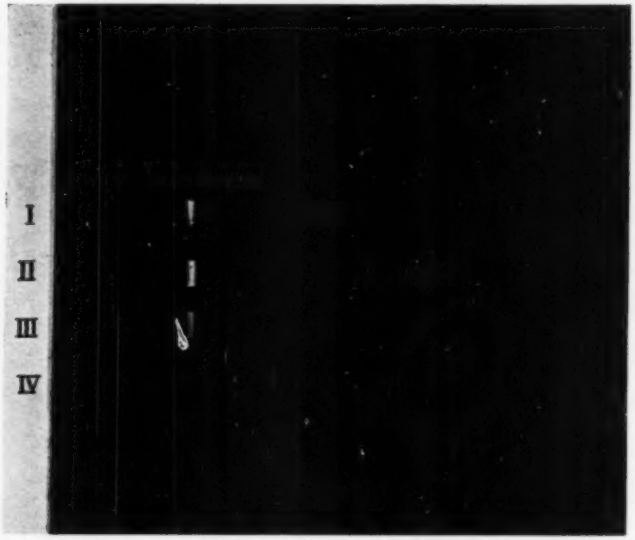

FIG. 6.—Fluorescence spectra of compounds derived from petrol exhaust soot: (excitation at 365 mμ).

I 3,4-Benzofluoranthene; II 1,2,3,4-Dibenzopyrene;
III 1,2,4,5-Dibenzopyrene; IV 1,12,2,3-Dibenzoperylene.

per million of petrol soot and 14 parts per million of diesel soot. The 1,2,4,5-dibenzopyrene occurred in the petrol soot at a concentration of about 10 parts per million.

DISCUSSION

3,4-Benzofluoranthene, tetracene, 1,2,3,4-dibenzopyrene, pentaphene and 1,2,9,10-dibenzotetracene, here recorded as occurring in vehicular exhausts, have been reported by Wynder and Wright (1957) as present in cigarette smoke condensates. The present author (1958) has also detected and estimated 1,2,3,4-dibenzopyrene in cigarette smoke.

3,4-Benzofluoranthene is the most recent hydrocarbon and the first fluoranthene derivative found to be carcinogenic. According to preliminary results obtained for mouse skin by Wynder in New York (Wynder, personal communication)

the compound is not much below 3,4-benzopyrene in carcinogenic potency, producing tumours in 100 per cent of the animals at 0·5 per cent concentration and even at 0·1 per cent is expected to produce lesions in the majority of animals.

1,2,4,5-Dibenzopyrene shares the property exhibited by the 1,2,3,4-, 3,4,8,9- and 3,4,9,10-derivatives of being a potent carcinogen (Arbuzov and Grechkin, 1952, 1953). In so far as is known, this is the first occasion on which the presence of 1,2,4,5-dibenzopyrene and 1,12,2,3-dibenzoperylene in any pyrogenic material has been shown.

SUMMARY

Fractions isolated from petrol engine and diesel engine exhaust samples were assayed for the presence of polycyclic aromatic hydrocarbons. The fractions examined had all succeeded 3,4-benzopyrene in initial chromatographic runs.

The methods employed were repetitive adsorption chromatography on alumina and silica gel, followed by ultra-violet absorption and spectrographic analysis of the eluates. By these methods the following compounds were identified in the petrol engine exhaust sample: 3,4-benzofluoranthene, tetracene (naphthacene), pentaphene, 1,2,3,4-dibenzopyrene, 11, 12-benzofluoranthene, 1,2,9,10-dibenzotetracene, 1,2,4,5-dibenzopyrene, and 1,12,2,3-dibenzoperylene. In the diesel engine exhaust sample, the following compounds were identified: 3,4-benzofluoranthene, pentaphene, 1,2,3,4-dibenzopyrene, 11,12-benzofluoranthene and 1,2,9,10-dibenzotetracene.

Of the above listed compounds, the 3,4-benzofluoranthene, 1,2,3,4-dibenzopyrene and 1,2,4,5-dibenzopyrene are carcinogenic, while the 1,12,2,3-dibenzoperylene, it is believed, has not yet been tested. This is the first time, it is thought, that the latter two compounds have been identified in any pyrogenic material.

I wish to thank Dr. P. R. Peacock, Director of Research, for helpful advice, and Dr. E. Clar, Chemistry Department, University of Glasgow, for the gift of samples of polycyclic aromatic hydrocarbons.

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IN VITRO UPTAKE OF ^{14}C LABELLED GLYCINE BY THE CELLS OF PLEURAL AND PERITONEAL FLUID

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It has often been observed that malignant tumours are able to obtain nourishment for growth while the tissues of the host waste away. The observations of LePage *et al.* (1952) on rats bearing transplanted Flexner-Jobling carcinoma indicate that the tumour grows by using amino-acids derived from the breakdown of normal tissues.

Many experiments have been performed to elucidate the mechanism and significance of amino-acid uptake by normal and malignant tissues. The complex problems involved have been recently reviewed by Campbell (1958). Regarding the difference, if any, between tumour and normal tissue, it can be said that *in vivo* experiments have on the whole failed to show any greater uptake of labelled amino-acids by tumour than by the other tissues studied such as liver and kidney, and this has lent support to the view that the priority of tumours may be due to a relative inhibition of their protein catabolism (Heidelberger, 1953).

In vitro, on the other hand, tumour tissue has been shown to take up labelled amino-acids in larger amounts than normal tissue. This phenomenon has been observed in the case of rat hepatoma, (Zamecnik *et al.*, 1948 ; Zamecnik *et al.*, 1951), mouse mammary carcinoma (Winnick, 1950), Ehrlich carcinoma (Christensen and Riggs, 1952 ; LePage, 1953), Gardner mouse lymphosarcoma (LePage, 1953) and Walker 256 rat carcinosarcoma (Nyhan and Busch, 1957). The discrepancy between *in vitro* and *in vivo* behaviour has been attributed to the poor blood supply of tumours (Zamecnik *et al.*, 1951), but Rotherham and co-workers (1957) have found that rat hepatoma shows a greater uptake of amino-acids into histones *in vivo* than normal liver, which would not be expected if the blood supply was inadequate.

In order to determine whether the *in vitro* increase in amino-acid uptake is a widespread property of human tumour cells, we studied the uptake of ^{14}C labelled glycine by the cells of pleural and peritoneal effusions, both benign and malignant, by the stripping film autoradiographic technique of Doniach and Pelc (1950).

Serous effusions are particularly convenient for this purpose for several reasons ; firstly, the cells are present in a viable state, and both mesothelial cells and tumour cells are able to enter mitosis in this medium *in vivo* ; secondly, the cells being investigated are sometimes present in the free state (although most types of tumour cells are more commonly found in small aggregates) ; and thirdly, the specimens are easily obtained in a sterile condition and without any admixture of mucus, necrotic débris or other confusing material. In all the specimens described the cells concerned were readily identifiable (Spriggs, 1957) ;

where doubt existed about the identification of the cells, the experiment has not been included.

MATERIALS AND METHODS

Eleven specimens from 10 cases have been included in the series being reported. Each specimen was citrated at the time of collection (1 : 10 volume of 3·8 per cent sodium citrate), and was used as soon as possible afterwards, and always within 24 hours. When received in the laboratory, it was centrifuged until the cells were deposited (1500 r.p.m. 10 mins.). The supernatant fluid was used as culture medium; this was thought to minimize any unphysiological alterations in the extracellular milieu. When red cells were scarce or absent, enough Group O red cells (previously washed in normal saline) were added to permit of subsequent satisfactory handling. A sufficient quantity of the supernatant fluid was discarded to give a cell count of about 5000 to 10000 nucleated cells per c.mm. 2 μc. [2-^{14}C]glycine (specific activity 4 μc/μMole) was added to 2 ml. concentrated cell suspension and the mixture was incubated at 37°C. for 3 hours. The cells were then deposited by centrifuging and air-dried films were made on glass slides. The remainder of the procedure was that described by Lajtha (1954), the exposure time being 10 days. Grain counts were performed on the selected cell types which were fully identifiable in the counterstained films. (Several films were discarded either because of difficulties in cell identification, or when there was no uptake presumably because of loss of vitality of the cells.) By means of an eyepiece attachment with ruled squares, the grains were counted per unit area inside the cell boundaries of a number of typical cells of each variety, and the mean values calculated. The background grain count per unit area was also obtained, and subtracted from each of the counts made over the cells. (The average background grain count for the whole series was 5·7 per 100 μ^2).

RESULTS

The results of the grain counts are shown in Table I (p. 134).

DISCUSSION AND CONCLUSIONS

In the fluids examined there was very little evidence of glycine uptake by the leucocytes, macrophages, or degenerate mesothelial cells (the latter are those which have lost their cytoplasmic basophilia). There was, on the other hand, a strikingly high uptake by the mesothelial cells with basophilic cytoplasm; their amino-acid uptake is presumably a reflection of their growth activity.

Tumour cells were examined in eight of the eleven fluids, and were invariably found to show more glycine uptake than the leucocytes. Their activity in this respect was not greater than that of basophilic mesothelial cells present in the same specimens, and the grain counts obtained were in general of the same order or lower. In case 3, two figures are given for the grain counts over tumour cells; the higher is for cells showing cytoplasmic basophilia and the lower for the cells with pale-staining cytoplasm. In case 10 the "oat-cells", which have only a small rim of cytoplasm and little basophilia, gave low grain counts. There appears, therefore, to be a correlation between grain-counts and basophilic staining (due to ribonucleic acid) both in mesothelial cells and in tumour cells.

TABLE I.—*Grain Counts Above Background per* 100 *sq. μ of Cell Surface*

Case No.	Diagnosis	BMC.	DMC.	M.	L.	N.	E.	TC.
1	Bronchopneumonia	55·0	7·4	..	5·4*	..	..	None found
2	Ca. uterus	26·2	..	..	4·8	..	..	„ „
3	Ca. ovary	69·2	..	..	2·3	0·1*	..	3·5 and 22·(
4	Ca. stomach	..	..	..	..	..	..	128·4
Right 4		..	..	..	..	..	..	71·6
Left 5	Ca. stomach	103·1	..	7·2*	..	..	−3·1*	98·3
6	Hodgkin's disease	..	..	6·8*	..	..	−0·5*	24·0
7	Ca. ovary	..	..	..	..	..	..	71·0
8	Ca. breast	..	..	..	..	..	..	15·5
9	Carcinomatosis ? primary	54·4	..	..	6·1	5·0	..	None found
10	Oat-cell ca. lung	13·6	2·2	..	0·2*	..	..	8·5

Explanations of abbreviations—

BMC. = basophilic mesothelial cells.
DMC. = degenerate mesothelial cells.
M. = macrophages.
L. = lymphocytes.
N. = neutrophil polymorphonuclears.
E. = eosinophil polymorphonuclears.
TC. = tumour cells.
* = total grain count less than 2 × that of background (i.e. no significant evidence of uptake).

The terminology is that of Spriggs (1957).
All the tumour cells were of familiar types except in case 6 (Hodgkin's disease), whose pleural effusion contained many bizarre cells like those of a reticulosarcoma.

Since the mesothelial cells and the tumour cells have very different origins, one is not comparing like with like; nevertheless in this situation there is no clear difference in glycine uptake between malignant and certain non-malignant cells, and the high uptake rate of malignant cells can be attributed to their state of active growth rather than to any fundamental metabolic difference.

SUMMARY

In vitro uptake of ^{14}C labelled glycine was studied in the cells of serous effusions from 10 cases by an autoradiographic technique. Tumour cells were present in 7 cases, and their glycine uptake was found to be much greater than that of leucocytes or macrophages. Mesothelial cells of the type with basophilic cytoplasm showed even greater uptake than tumour cells.

It is concluded that the glycine uptake in these conditions is correlated with the degree of cytoplasmic basophilia, and is independent of whether the cells are malignant or not.

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ON THE CORRELATION BETWEEN METABOLIC AND STRUCTURAL CHANGES DURING CARCINOGENESIS IN RAT LIVER

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THE nature and the origin of the malignant growth resulting from the introduction of a carcinogenic compound into the animal cell are still matters of conjecture rather than that of established causal relationship. An attractive theory was given by Warburg who explained the malignant growth as the result of damage to respiration. In his words "The interference with respiration is, from the standpoint of the physiology of metabolism, the cause of tumours. If the respiration of the growing cell is disturbed, the cell dies. If it does not die, a tumour cell results" (Warburg, 1926). This theory undoubtedly unifies various non-specific tumour-inducing factors into a single cause, and at the same time, is specific enough to allow biochemical testing. Nevertheless, Warburg's theory is opposed persistently by a group of investigators who maintain that respiration of tumours is neither qualitatively nor quantitatively changed compared with the normal (Weinhouse; 1951, 1956; Weinhouse, Millington and Wenner, 1951; Greenberg, 1955). These authors accept only a high level of fermentation, discovered by Warburg (1926), as the undisputed but so far unexplained, feature of tumours. The whole controversy was brought to a sharp focus in a recent series of discussions (Warburg, 1956; Weinhouse, 1956; Warburg, Burk and Schade, 1956).

The aim of the work presented in this paper was to elucidate the nature of the permanent damage to the cell exerted by carcinogenic compounds by correlating the metabolic and structural changes (as revealed by differential centrifugation) and by looking for a common denominator from which these changes are derived. We turned our attention from the beginning toward ribonucleic acid and its intracellular distribution as the factor possibly determining the course of events. We have seen previously (Fiala, Sproul and Fiala, 1956, 1957) that in certain target organs the stimulation by anterior pituitary hormones leads to a rapid synthesis of cytoplasmic, especially ergastoplasm-bound, ribonucleic acid, with an almost parallel increase of cell respiration. It seemed possible that if there is an interdependence between two classes of cytoplasmic constituents, the respiratory elements (mitochondria) and the RNA-rich ergastoplasm, as part of circular causal mechanism, this interdependence might play a most significant role also in carcinogenesis. The consequent work warranted this assumption. As the next step we investigated how the increased fermentation is correlated with the intracellular distribution of ribonucleic acid. Finally we investigated how the metabolic and structural changes are correlated with the cell proliferation and the onset of malignancy.

MATERIALS AND METHODS

Sprague-Dowley albino rats and Swiss albino mice were used as experimental animals. The rats were of both sexes, weighing in average 180 g. at the start of the experiments. Controls were fed a basal diet consisting of vitamin-free casein (16 per cent), dextrose, corn oil, a mixture of mineral salts in appropriate amounts and a low level of riboflavin, as recommended by Miller *et al.* (1948). Experimental animals received 600 mg. of carcinogen per kg. of food.

The carcinogen, 3′-methyl,4-dimethyl aminoazobenzene was synthesized and crystallized from alcoholic solution by water. Its identity was established from absorption spectra (max. at 510 mμ at pH 2·3) and by measuring the melting point (119°). The animals were sacrificed at appropriate time intervals and compared with controls fed for the same period of time and, whenever possible, of similar nutritional state. The latter condition is important because it is known that protein depletion affects not only the weight of the liver but also its intracellular composition (Lagerstedt, 1949; Muntwyler, Seifter and Harkness, 1950). The chilled tissue was homogenized in a Potter-Elvehjem homogenizer with cold isotonic sucrose. Differential centrifugation was applied essentially as in our earlier work (Fiala *et al.*, 1956). Four subcellular fractions were separated and designated as "N" (nuclei), "M" (mitochondria), "R" (containing the broken basophilic ergastoplasm) and "S" (supernatant remaining after two hours centrifugation at 25,000 *g* in a Servall SS-1 centrifuge) or "S*" (supernatant remaining after 1 hour at 59,000 *g* in a Spinco preparative ultracentrifuge). No effort was made to separate the "fluffy layer" ("CH") which was included in the R fraction. The purity of the nuclear and mitochondrial fractions was checked microscopically using a freshly prepared mixture of methyl green and pyronin Y. Contamination by RNA-rich elements appears as a pink coloration easily seen even at low magnification (× 450) while purified mitochondria do not stain. DNA and RNA were extracted by Schneider's method (Schneider, 1945) from homogenized tissue or from separated fractions and their amounts determined colorimetrically, using diphenylamine (DNA) and orcinol (RNA) reactions. Protein nitrogen was determined by nesslerization from the residuum after nucleic acid extraction. RNA/Protein nitrogen is referred to as the "basophilic quotient" (B.Q.).

Manometric measurements were performed in a Warburg apparatus at 37° Succinoxidase activity of isolated mitochondria was measured in an air phase in sucrose suspension buffered with potassium phosphate (0·5 ml. of isotonic buffer for the total volume of 2·5 ml). Succinoxidase activity of tissue slices was measured in a Ringer solution, buffered to pH 7·8 and in a 100 per cent O_2 atmosphere. The side arm in both cases contained 0·2 ml. of 0·5 M sodium succinate and 3 mg. of Cytochrome c in 0·2 ml. of saline. In order to measure the respiration of mitochondria, the purified fraction spun down from sucrose suspension by centrifugation for 7′ at 25,000 *g*, was resuspended in a modified Ringer solution containing fortifying factors as used by Wenner, Spirtes and Weinhouse (1951). Sodium pyruvate (8×10^{-3} M) served as substrate. Anaerobic glycolysis in tissue slices was determined by measuring lactic acid production. Tissue slices were incubated in a Warburg water bath at 37° for two hours in a Ringer solution containing 0·2 per cent dextrose and saturated with 95 per cent N_2, 5 per cent CO_2 for 15 minutes. The stream of this gas mixture was led through two washbottles contain-

ing Fieser's solution (Fieser, 1941), in order to remove all traces of oxygen and through a washbottle containing a saturated solution of lead acetate to remove traces of H_2S. Lactic acid was determined according to the method of Barker and Summerson (1941) and measured colorimetrically at 565 mμ. in a Beckman spectro-photometer from the aliquots of acid extracts after precipitating both the unincubated (O′ control) and the incubated (2 hours) samples with equal volume of 10 per cent trichloracetic acid. In the resuspended sediments DNA was determined colorimetrically and the lactic acid content of each extract related to the amount of DNA in the sediment. The results were expressed on the basis of units of DNA (5×10^{-12} g.) as μg. of lactic acid produced by the amount of tissue containing 100×10^6 such units of DNA. The reasons which prompted us to use this standard amount of DNA as a reference for expressing metabolic or cell fractionation data will be mentioned at the end of this section.

For the study of changes in the levels of non-protein sulphydryl groups the polarographic method was chosen because no drastic procedure is employed in this determination. In order to follow the level of reduced glutathione, 3 g. of tissue were homogenized in 10 ml. of Ringer solution and directly polarographed in a N_2 atmosphere at room temperature. It has been shown (Hoščálková, Černoch and Šantavý 1955) that GSH can be determined directly in an homogenate when the dropping mercury electrode serves as anode. The depolarization potential is at $+0{\cdot}5$ v in respect to the normal calomel electrode (Laitinen and Sullivan, 1941). After anodic curves were recorded the remainder of the homogenate was diluted with three volumes of Ringer solution and centrifuged for 1 hour at 25,000 *g* in the cold. To the supernatant an equal volume of 5 per cent sulphosalicylic acid was added and the precipitate removed by centrifugation. Two ml. of the deproteinized sample were diluted up to 10 ml. with distilled water. 0·4 ml. of this solution was added to the mixture of 5 ml. of 0·1 N NH_4Cl in 0·1 N ammonia and 0·5 ml. of either 1×10^{-3} M $CoCl_2 \cdot 6\,H_2O$ or 1×10^{-3} M $Co(NH_3)_6Cl_3$ (luteocobaltichloride). These are polarographic media in which compounds containing free SH- and SS-groups show a catalytic increase of the cathodic current (Brdička, 1933; Kolthoff and Lingane, 1952). The proteins show a characteristic "double wave" in both these media, while the lower molecular compounds such as cysteine, cystine and glutathione (both GSH and GSSG) give a single maximum and only in the bivalent cobalt solution (Brdička, 1933). The polarograph was the KLB-Blomgren, type 3266, attached to a Leeds-Northrup automatic recorder (Speedomax type G). Anodic curves were recorded at the sensitivity of 10 microamperes, cathodic curves at 50 microamperes for full scale deflection.

For polarographic work only liver tissue perfused with Ringer's solution *in vivo* was used.* For comparison Novikoff rat hepatoma was also included.† The tumour contained, usually, much blood. The homogenized tumour was, therefore, centrifuged at a low speed and a great part of the red blood cells sedimented.

In a smaller number of experiments, painting mice with 3,4 benzbyrene in 0·5 per cent benzene solution on hairless skin of 1–2 days old mice was applied. The mice were sacrificed 24 hours later. The presence of carcinogen was obvious by its strong characteristic fluorescence when the animals were illuminated in the

* Miss Nancy Tracy assisted in these experiments.

† Obtained through the courtesy of Dr. A. B. Novikoff of the Albert Einstein Medical College, New York.

dark with UV-light. The epidermis was sliced with Stadie's hand microtome (Stadie, Riggs and Haugaard, 1945).

Rapid protein and cytoplasmic RNA syntheses occurring in the adrenal cortex and ovaries of the rat after injection of ACTH and FSH (Armour & Co., Chicago) were observed under conditions previously described (Fiala *et al.*, 1956, 1957).

The expression of metabolic and fractionation data on the basis of DNA content of the normal diploid nucleus was adopted in this work from the following reasons: It has a deeper physiological meaning than the expression on the " per cell " basis, although the latter first suggested by Davidson and Leslie (1950) was already a great advance over the older procedure of expressing data on a dry or wet weight basis. There are, however, several factors which devaluate the " per cell " basis as a reference for metabolic and fractionation data, except when dealing with pure cellular sublines or clones in tissue cultures. Not only are the organs usually composed of several different cell types so that we relate our measurements to an abstract "average cell ", but many cells such as those in liver are binucleate and their percentage in an organ may vary. There are also many polyploid nuclei, especially in tumours. The total DNA content of the tissue can thus hardly be utilized in the estimation of the number of cells when divided by the DNA content of the cell nucleus nor can the counting of nuclei in an aliquot serve this purpose. Unless we count the number of cells directly, as is possible with the Ehrlich ascites tumour, we have no right to interprete results on " per cell " basis. On the other hand the ratio : total DNA of the tissue/DNA content of normal diploid nucleus, referred to as number of DNA units of tissue, has a physiologic meaning. First, the amount of DNA in the diploid set of chromosomes is constant and characteristic for each species. Second, normal cells when containing an increased amount of DNA, either due to binucleate forms or polyploidy, have correspondingly increased cytoplasm. Significantly, in tumours this rule is not obeyed. For these reasons the DNA content of diploid nuclei which in the rat is 5×10^{-12} g. (Vendrely, 1955; Thomson *et al.*, 1953; Fiala *et al.*, 1956) will be used throughout this work as the basis (" DNA unit ") for expressing metabolic and fractionation data.

RESULTS

1. *The respiratory damage during carcinogenesis*

The development of gross tumours in rat liver induced by feeding with 3'-Me, 4-DAB takes usually 150–200 days. When a comparison is made (1) on a dry weight basis (Q_{O_2}), (2) c.mm. O_2 consumed by an aliquot of tissue containing 100×10^6 nuclei, and (3) a DNA unit basis, between normal rat liver, tumour infiltrated liver 200 days after initiation of carcinogen feeding and Novikoff transplantable hepatoma, the results differ sharply (Table I). On a dry weight basis the difference in the rate of respiration between these tissues is not impressive, whereas on the nuclear basis it is much more pronounced. This is due to the fact that there are more nuclei per unit of weight in tumour than in normal tissue. Still more striking is the difference on a DNA basis. In Table I Ehrlich ascites tumour* is also included. The table illustrates how an apparently high respi-

* Obtained through the courtesy of Dr. Samuel Graff and Mr. Gerald Zagall of the Department of Biochemistry, Columbia University.

ration in Q_{O_2}-values of tumours dwindles when a physiologically significant basis of comparison is used and also that the Ehrlich ascites tumour closely approximates that of rat hepatoma. Corresponding-values for tumour infiltrated liver are intermediate between those for normal liver and transplanted hepatoma due to the mixture of normal and malignant cells.

TABLE I.—*Respiratory Activity of Normal and Tumour Tissue Slices.*

Tissue	Q_{O_2}	Q_{O_2} (Nucl. $\times 10^4$)	Q_{O_2} (DNA $\times 10^4$)
Normal rat liver	12·0	8·0	8·0
200 days 3′-Me-4-DAB	8·0	4·0	3·53
Novikoff hepatoma	9·8	1·6	1·25
Ehrlich ascites tumour	5·0	2·7	1·1

The obvious reduction of respiratory rate in 3′-Me, 4-DAB induced cancerous liver may have different reasons. It is known that most or all respiration is performed in mitochondria. The most plausible explanation for the reduction of respiration in carcinogen induced tumours seems to be a direct inhibition of respiration by accumulation of the carcinogenic compound in mitochondria. Yet, should this be the case, the same amount of mitochondria in units of protein nitrogen from the normal liver should consume more oxygen than the equivalent amount of mitochondria from tissue full of carcinogen or from tumour. Our experiments have shown, however, that the specific respiratory activity (c.mm. O_2 consumed per mg. of protein nitrogen) with Na pyruvate as substrate remains normal for mitochondria during feeding with carcinogenic azodye and even in the tumour after 200 days (Table II).

TABLE II.—*Respiratory Activity of Isolated Liver Mitochondria*

	c.mm. O_2	Prot.N. (mg.)	Spec. activity $\frac{\text{c.mm.}O_2}{\text{mg. prot. N.}}$
Control	−585	9·00	65
Experimental (46 days 3′-Me-4-DAB)	−475	6·75	70
Experimental (hepatoma)	−320	4·9	65

When the specific activity of a complex of respiratory enzymes such as succinoxidase was measured during carcinogenesis and in tumour mitochondria, it was seen again that no reduction of respiration per mg. of protein nitrogen occurred during this time, although in 50 days of carcinogen feeding the tissue was still very rich in protein-bound carcinogen as manifested by the intense red colour of the sediment after TCA precipitation (Table III).

TABLE III.—*Respiratory Activity of Isolated Liver Mitocho Succinoxidase, 50 days of diet 3′-Me, 4-DAB*

	c.mm. O_2	Prot. N. (mg.)	Spec. activity $\frac{\text{c.mm. }O_2}{\text{mg. prot. N.}}$
Control 1	−680	0·566	1207
Control 2	−635	0·524	1206
Exp. 3	−426	0·377	1129
Exp. 4	−419	0·341	1228

An analogous result was obtained when the respiration was measured in sliced epidermis of 2-day old hairless mice, 24 hours after painting the skin with benzpyrene.. The presence of the carcinogen in the skin was visible after illumination with an UV-lamp and at least some portion of the carcinogen was protein-bound as shown by liberation of fluorescent material after hydrolysis of separated cytoplasmic fractions. Nevertheless the benzpyrene painted mouse epidermis respired just as well as that of unpainted controls (Table IV).

TABLE IV.—*Respiration and Succinoxidase Activity in Benzpyrene Painted Mouse Epidermis*

	Experiment		Control	
Amount of tissue (mg.)	168	168	168	168
O_2 uptake in 30 min. (c.mm.)	98	95	95	93
Q_{O_2}	5·8	5·7	5·7	5·62
Succinoxidase, pH 7·4 O_2 uptake in 30 min. (c.mm.)	210	220	212	188
Q_{O_2}	12·7	13·2	12·8	11·4

The experiments just described have shown that mitochondria from normal liver, from liver during carcinogenesis and from developed tumours are equivalent, at least in regard to the rate of respiration per mg. of protein nitrogen. The same amount of mitochondria under these conditions consume the same amount of

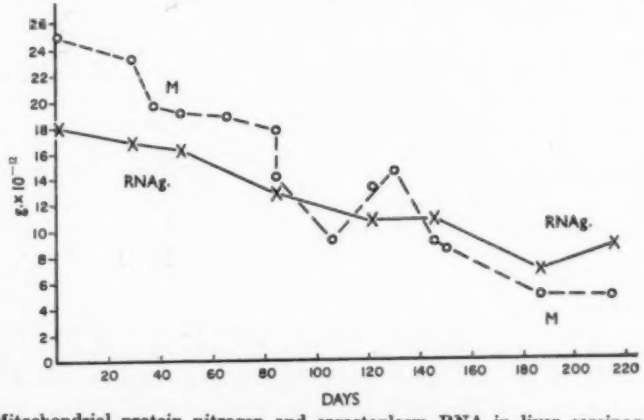

FIG. 1.—Mitochondrial protein nitrogen and ergastoplasm RNA in liver carcinogenesis.

oxygen. Damage to respiration during carcinogenesis must, therefore, be induced by the reduction in amount of mitochondria and not by a direct inhibition of the respiration. That this is the case is demonstrated by data in Fig. 1 which shows a depletion of mitochondrial elements in the course of 200 days of carcinogenesis. The results are expressed in pg. (1×10^{-12} g) per unit of DNA. The reduction in amount of mitochondria in liver tumours as compared with normal liver

has been established (Schneider *et al.*, 1953; Allard, Lamirande and Cantero, 1953; Fiala, 1953; Howatson and Ham, 1955). For a simple demonstration of this fact it is sufficient to compare the turbidities of fresh mitochondrial suspensions obtained from the same wet weight of liver tissue and of hepatoma. The suspension from normal liver appears dense, while that of hepatoma is completely transparent at the same dilution. A similar although not as pronounced difference appears soon after start of feeding carcinogenic azodye. Thus the respiratory damage occurring during carcinogenesis and in tumours is not due to respiratory inhibition but to the depletion of mitochondria.

2. *Correlation between mitochondria and ergastoplasma during carcinogenesis and after hormone stimulation*

Mitochondria are not the only elements in cells which are reduced in amount during carcinogenesis and in tumours. It has been observed earlier (Howatson and Ham, 1955; Fiala, 1953) that the amount of ergastoplasm in hepatoma is lower than in normal liver. When the ergastoplasm-bound RNA (sedimentable RNA after 1 hour at 59,000 *g* and resedimented for 2 hours at 30,000 *g*) was measured during 200 days of carcinogenesis and plotted against the mitochondrial protein (Fig. 1) it was seen that the depletion of both cellular components goes almost parallel.

This parallelity may be thought to result only from general depletion in cytoplasmic structures. On the other hand it may be a manifestation of a close interdependence between mitochondria and the ergastoplasm. The plausibility of the second alternative is indicated by an experiment with tropic anterior pituitary hormones. This experiment, unpublished so far, illustrates well the interdependence between mitochondria and ergastoplasm. It is summarized in Fig. 2.

As seen from the figure the amount of succinoxidase increased suddenly after a single injection of ACTH or FSH and reached a level some 60 per cent (ACTH) or 35 per cent (FSH) greater than the original, while in the same time, the ergastoplasm-bound RNA increased by 25 and 22 per cent respectively. The effects were greater after several repeated injections but occurred, judging from DNA content, without being accompanied by cell division. In general the increase in succinoxidase activity and in the mass of mitochondria accompanied the rise of ergastoplasm-bound RNA. The increase of ergastoplasm-bound RNA was manifested by a striking increase of the basophilic quotient of this fraction, from 1·1 to as high as 1·8 after several injections of the hormone.

It is the time-relationship which seems to mark the interrelationship between ergastoplasm-bound RNA and mitochondria-bound succinoxidase. As long as there was no change in the level of ergastoplasmic RNA there was also no increase in the level of succinoxidase. The moment, however, the RNA in the ergastoplasm was synthesized, the succinoxidase activity increased and continued to increase as long as there was a new synthesis of RNA in the ergastoplasm. It stopped when the synthesis of RNA ended. The regularity of this process resembled the light in an electric bulb following turning on the switch. These reverse effects, namely the increase of respiration (and mitochondria) related to the increase of ergastoplasm-bound RNA in hormonal stimulation and the gradual slow inhibition of both during carcinogenesis provide the evidence for the close interdependence of these cytoplasmic structures.

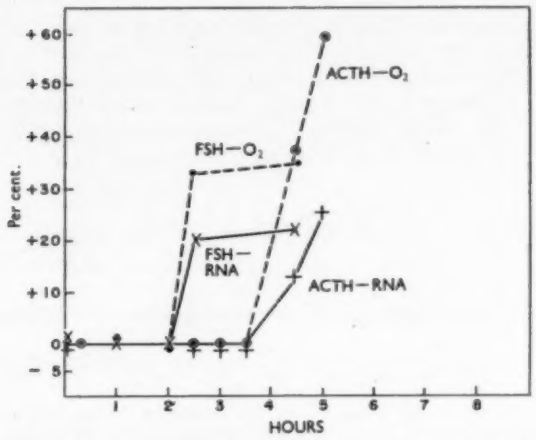

FIG. 2.—Dependence of Succinoxidase Activity on Cytoplasmic RNA Stimulated by FSH and ACTH.

A. Ten female rats (60 days old, 200 g.) used for FSH experiment. Two controls, the rest injected each with 2 mg. of purified hormone. Manometric measurements performed 1, 2, 2½ and 4½ hours after hormone administration. RNA determined from the same samples.

B. Twelve male rats used for ACTH experiment. Two controls, the rest injected each with 25 USP units of ACTH Armour. Manometric measurements and RNA determinations performed at 0, 2½, 3, 3½, 4½ and 5 hours after injection.

3. *Fermentation and its correlation to the distribution of cytoplasmic ribonucleic acid during rat liver carcinogenesis*

When anaerobic glycolysis was measured at various time intervals and compared with liver tissue from controls fed basal diet only, it was seen that there was a lag period of 80–100 days during which the experimental animals did not differ from controls. As seen from the simultaneous measurements of succinoxidase activity, there was a considerable loss in activity of this enzyme complex during this period due to the depletion of mitochondria. Approximately 80–100 days after initiation of carcinogen feeding a sudden increase of anaerobic glycolysis could be observed. From this time the increase of anaerobic glycolysis proceeded uninterruptedly up to the development of gross tumours in liver (Fig. 3). (The recently repeated series of succinoxidase determinations confirmed that the levels of this respiratory enzyme complex are essentially lower in experimental samples than in corresponding controls. In several instances, however, the succinoxidase at the time when the glycolysis started to increase, differed from the controls less than in other cases where the glycolysis was still unchanged. It has to be decided by further experiments whether this is due only to different individual sensitivity to respiratory depletion or whether this has a deeper meaning.)

In further experiments we investigated whether this sudden increase of anaerobic glycolysis is accompanied by any conspicuous change in the distribution of cytoplasmic RNA. We measured, therefore, in various stages of the process the ratio between the ergastoplasm-bound RNA (and whatever RNA may be found in mitochondria) against the RNA remaining in the supernatant (S*). The experiments are summarized in Table 5.

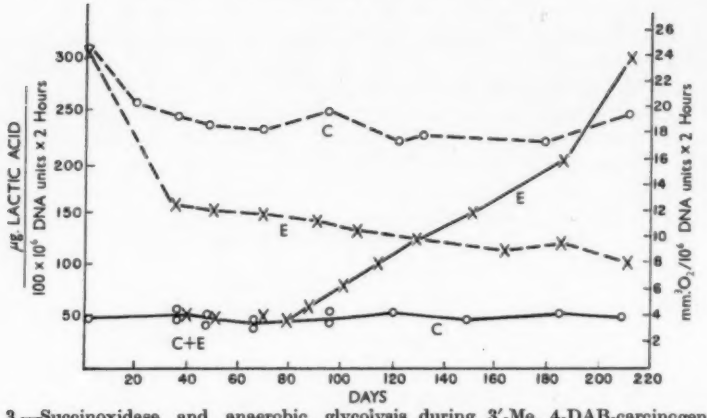

FIG. 3.—Succinoxidase and anaerobic glycolysis during 3'-Me, 4-DAB-carcinogenesis. Two upper curves relate to oxygen uptake of succinoxidase in the control (C) and experimental sample (E). Lower curves refer to anaerobic glycolysis in control and experimental.

TABLE V.—*Distribution of Cytoplasmic RNA during Carcinogenesis*

Days	Non-sedimented / sedimented RNA × 10⁻¹² g: Control	Experiment	Anaerobic glycolysis
0	$\frac{7\cdot4}{24\cdot0}=0\cdot3$	—	—
50	$\frac{4\cdot6}{14\cdot0}=0\cdot33$	$\frac{4\cdot2}{12\cdot0}=0\cdot35$	0
121	$\frac{4\cdot0}{12\cdot0}=0\cdot33$	$\frac{4\cdot8}{11\cdot0}=0\cdot44$	100%
130	$\frac{4\cdot6}{23\cdot0}=0\cdot202$	$\frac{3\cdot9}{14\cdot4}=0\cdot27$	0
146	—	$\frac{5\cdot5}{11\cdot0}=0\cdot5$	150%
187	$\frac{5\cdot5}{16\cdot2}=0\cdot31$	$\frac{6\cdot0}{7\cdot3}=0\cdot82$	300%
215	—	$\frac{7\cdot3}{8\cdot9}=0\cdot84$	500%
Hepatoma (rat)	—	$\frac{10\cdot6}{10\cdot0}=1\cdot06$	1200%
Ehrlich ascites tumour	—	$\frac{12\cdot5}{2\cdot5}=5\cdot0$	3000%
Mouse sarcoma	—	$\frac{8\cdot7}{4\cdot0}=2\cdot2$	—
Mouse mammary adeno Ca	—	$\frac{5\cdot4}{4\cdot2}=1\cdot3$	—

It can be seen that with the sudden increase of fermentation a change in distribution of cytoplasmic RNA takes place in the cell in such a way that the relative proportion between sedimented (ergastoplasm plus mitochondria) and non-sedimented RNA shifts in favour of the latter. In developed tumours this excess of

non-sedimented over the sedimented RNA was still more striking. In the last columns of Table V three mouse tumours are also listed. It can be seen that they display the same or even greater preponderance of non-sedimented RNA over the sedimented cytoplasmic RNA fraction. The converse was also seen ; when on one occasion no increase of fermentation occurred despite 130 days of feeding with azodye, there was no increase in the relative proportion of the non-sedimented RNA fraction.

4. *Intracellular changes in levels of polarographically active sulphydryl groups*

The onset of increased anaerobic glycolysis characterizes thus an important turning point in the process of carcinogenesis. In an attempt to characterize further the two stages of carcinogenesis we studied the distribution of free, non-

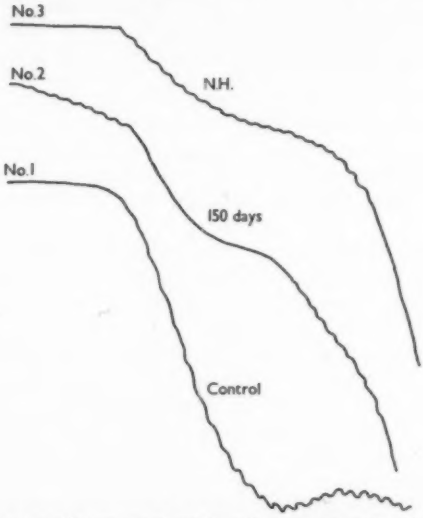

FIG. 4.—Polarographic (anodic) waves of rat liver homogenates showing the drop in the level of GSH of the experimental sample.

protein, polarographically active, SH and SS groups. Fig. 4 illustrates the great reduction in the level of GSH in rat liver homogenate after 5 months of feeding with 3′-Me,4-DAB. This reduction is even greater in Novikoff hepatoma when compared with the GSH level at the onset of the carcinogenic diet.

The time changes in the levels of GSH during carcinogenesis are shown in Table VI. It may be seen that diet alone did not have any significant influence nor did the presence of carcinogen affect the level of GSH up to the period of approximately 80–100 days after the start of the feeding. After this period, however, a rather sudden drop in GSH levels per unit of DNA occurred. The developed liver tumours and Novikoff hepatoma contained only some 20 per cent of the normal liver GSH.

A completely different result was obtained when the total amount of non-protein sulphydryl groups was estimated polarographically by using the catalytic

TABLE VI.—*Polarographic Test for GSH in Homogenized Liver during Carcinogenesis*

Tissue	Normal liver	Liver fed basal diet		Liver fed 3'-Me-4-DAB								Novikoff hepatoma
Time (days)	—	30	180	30	60	80	100	110	150	180	210	—
Value (cm.)	10·8 (5 determ.)	9·9	8·4	11·9	12·0	11·0	7·0	5·4	3·8	4·0	2·56	1·34 (5 determ.)

These data refer to height in cm. of polarographic anodic wave $2GSH \rightarrow GSSG + H_2$ as shown directly in the homogenate containing approximately 100×10^6 units of DNA in 1 c.cm.

effect of SH- (and SS-groups) on the dropping mercury electrode in the presence of ammoniacal solution of cobalt. When a solution of trivalent cobalt, which does not react with low molecular sulphydryl groups, was used, no significant difference was seen between controls and experimental samples, indicating that

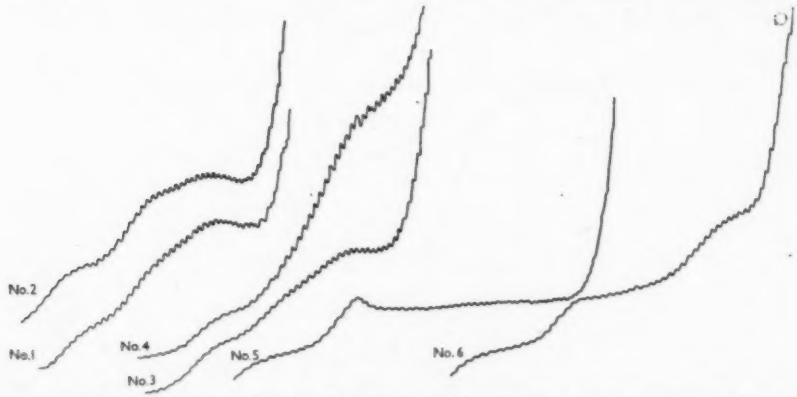

FIG. 5.—Effect of carcinogenesis (60 days feeding 3'-Me-4-DAB) on free, polarographically active, sulphydryl groups in the supernatant of liver.

1. Control (basal diet)
2. Experimental

0·2 ml. supernatant (dilution 1 : 10) added to Brdička's solution containing trivalent cobalt ($Co(NH_3)_6Cl_3$). No difference in polarographically detectable SH-content of soluble proteins.

3. Control (basal diet)
4. Experimental

Idem but Brdička's solution contains bivalent cobalt ($CoCl_2$). Experimental sample shows increase of non-protein SH-group.

5. Control (basal diet)
6. Experimental

Effect remains in deproteinized (sulfosalicylic acid) samples.

the amount of proteoses (or possibly also mucoproteins) is approximately the same in both cases. On the other hand a striking effect was seen in experimental samples when the same test was performed using bivalent cobalt. In this case the catalytic current at the cathode showed a much higher catalytic wave in the experimental sample than in the control, regardless whether the test was done with liver a month after start of carcinogen feeding or with tissue containing gross tumours. An illustration of this test is shown in Fig. 5.

The height of the catalytic SH-wave of the deproteinized supernatant was seen to increase very soon after the introduction of carcinogen. This effect is the more striking as, on the whole, the protein content of this fraction lags behind controls in the absolute amount. The effect disappeared for the most part when the supernatant was preincubated with monoiodoacetic acid (4 mg. in total volume of 3 ml.) at slightly alkaline pH for 30 minutes. The increased catalytic current at the cathode is undoubtedly due to the increased total level of sulphydryl (SH, not SS) groups as the result of carcinogen feeding.

5. *Proliferation and the onset of malignancy during carcinogenesis in liver*

As shown in the preceding sections there occurs during carcinogenesis a gradual depletion of cytoplasmic structures. The results represent only a statistical picture of an effect on a heterogenous population of several kinds of cells from which the liver consists. It might be that already in the first stage before the increased glycolysis appears; many of the parenchymatous liver cells die and are replaced by cells poor in cytoplasmic structures, *e.g.* by cells of proliferating biliary ducts. On the other hand it might be also that the dedifferentiation is not necessarily due to the inability of the cell to replace the existing subcellular elements damaged by a carcinogen in a numerically unchanged population of cells, but may result from premature divisions induced by the carcinogen before the cytoplasmic content of cells and their volumes doubled (Needham, 1952; Berglas, 1957).

One may assume that if either of these explanations fitted the actual course of events, the cell population during carcinogenesis would not remain numerically constant but would undergo very conspicuous variation. On the other hand if no such variation for a given period of time is observed, one has to assume that the dedifferentiation described and so easily detected by differential centrifugation is due to processes occurring within a great majority of the cells in relatively stable cell population. The process of reparation and proliferation, although conspicuous to a pathologist by histological methods, could apply only to a minority of the cell population of the whole organ which could not account for gross cytoplasmic depletions detected by differential centrifugation.

Table VII correlates the total amount of DNA in liver (expressed in DNA units) with liver weight during the whole period of carcinogenesis. It was seen that whereas liver weight is subject to great fluctuation, the DNA amount is practically constant for at least 75 days, that is, for the period of time during which gross changes in cellular composition, from the viewpoint of differential centrifugation, took place.

A massive proliferation of cells was detected only in the second period of carcinogenesis, after or simultaneously with the increased glycolysis. The relatively stable cell population during first stage of liver carcinogenesis indicates, therefore, that the depletion of cytoplasmic structures in the organ does not stem to any greater extent from the replacements of one cell type by another nor from premature cell divisions but mostly from a process affecting the bulk of the organ.

DISCUSSION

The data obtained seem to establish two distinct stages in the process of liver carcinogenesis induced by 3′-Me, 4-DAB. In the first stage, which takes about half

TABLE VII.—*Liver Weight and Cell Proliferation during 3'-Me, 4-DAB Carcinogenesis*

Days		0	11	19	21	44	50	64	71	75	100	119	192
g Liver weight	C	6·85	—	3·20	5·6	5·56	4·73	4·2	8·5	5·5	6·0	6·85	—
	E	—	6·6	5·39	5·65	3·8	6·25	5·3	7·68	6·5	9·28	18·3	11·85
% Body weight	C	4·16	—	2·7	2·9	4·5	3·2	3·5	3·3	2·8	3·3	3·3	—
	E	—	4·2	3·7	4·0	3·3	4·4	4·2	4·2	3·9	5·8	8·0	7·3
DNA units $\times 10^4$	C	3,200	—	3,175	3,426	3,100	3,100	2,340	3,260	3,596	3,300	3,000	—
	E	—	3,380	4,140	3,400	3,100	3,071	3,230	3,880	3,819	6,000	13,730	5,250
Anaer. glycog.*	C	—	—	—	—	—	—	—	—	—	—	—	—
	E	0	0	0	0	0	0	0	+20%	+50%	+100%	+200%	+250%

* Excess over the control.

Note: Animals for this experiment were chosen with regard only to length of feeding regardless of body weight.

of the time span of the whole process, the cell suffers respiratory damage due to the gradual depletion of mitochondria and organized ergastoplasm. The carcinogen is not a respiratory inhibitor and the respiratory damage is, therefore, only a secondary effect of carcinogen binding.

The second stage is characterized not only by a relatively sudden increase of glycolysis supplementing the insufficient respiration but also by a sudden change in the distribution of non-protein sulphydryl groups and, finally, by a redistribution of cytoplasmic ribonucleic acid. The circumstance that all these changes occur — within the limits of observation — at the same time, is apparantly significant. If we look upon the animal cell as an Homeostat (Fiala, 1958) we may say that these variables behave as step functions suddenly changing their values when the system reaches its critical state (Ross Ashby, 1952). By entering a new equilibrium, marking malignant transformation, the cell solves its problem of adaptation.

The parallelity of effects on mitochondria and ergastoplasm obtained by application of anterior pituitary hormones and by application of carcinogen suggests that both these cytoplasmic structures depend on a common factor which is influenced in opposite ways by hormone and by carcinogen.

The basic fact, from which all observations reported in this work originated, was the scarcity of mitochondria in liver tumours. The question must, therefore, arise: Can the carcinogenic process be considered as resulting always from the depletion of mitochondria and ergastoplasm so that the observations here reported may have general validity or are they pertinent only to liver carcinogenesis? It has been reported (Mateyko and Kopac, 1956) that ovarian tumours are marked by a negligible amount of peripheral cytoplasm, as compared with normal ovarian controls, which themselves have relatively little cytoplasm. Even in the giant Sternberg cells of Hodgkin's lymphoma the cytoplasm contains very few mitochondria and little of the endoplasmic reticulum (ergastoplasm) as observed by electron microscopy (Frajola *et al.*, 1956); in contrast enormous multilobal nuclei are present. A striking depletion of cytoplasmic structures in precancerous and cancerous skin was also observed with electron microscopy (Albertini, 1952,

1953). The depletion of mitochondria and of ergastoplasm is thus a characteristic feature of tumour cells.

It has been argued (Laird, 1954) that in certain tissues such as pancreas or lymph nodes the amount of mitochondria is also low and that it is incorrect, therefore, to ascribe any importance to the small quantity of mitochondria in tumours. This criticism is invalid because it is meaningless to compare pancreas with hepatoma ; pancreas should be compared with pancreatic tumour and lymph nodes only with lymphoma. Similarly it has been claimed (Schmidt and Schlief, 1955) that the cells of Ehrlich ascites tumour contain a large amount of porphyrins and cytochromes and demonstrate high Q_{O_2}-value. We have demonstrated, however, that the respiration is actually very low when expressed on the basis of DNA units (Table I). Also the fractionation of these cells (Fiala, 1958*b*) convinced us that in this tumour the mitochondria are scarce in proportion to DNA content.

In special instances it may be wrong to assume that the homologous normal tissue is the adequate control of a tumour. Thus by comparing the respiration of normal mouse epidermis with skin tumours it was concluded (Carruthers and Suntzeff, 1947) that tumour growth leads to an increase of succinoxidase activity and a greater respiration. Notwithstanding the fact that any comparison must be done on a per cell basis, or, still better, on a unit of DNA, one must point out that all the normal epidermal cells are not the natural counterpart of the skin tumour. Much of the epidermis consists of very differentiated cells the cytoplasm of which is almost completely filled out with tonofibrils with only few remaining preformed cytoplasmic elements. These cells are keratinized and, consequently, have a low respiration. There the loss of metabolic function is the result of normal differentiation in order to assume a specialized protective function. Skin tumours, on the other hand, stem from the cells which preceded normal differentiation. It is from the basal cells that both normal differentiation and malignant transformation stem and with which the only valid comparison can be made. It seems, thus, that all main objections against the thesis of mitochondrial depletion in tumours can be answered satisfactorily.

In contrast to our finding of sudden increase in anaerobic glycolysis, Nakatani *et al.* (1936) observed this increase right after the initiation of carcinogen feeding. These findings were not confirmed by Orr and Stickland (1941) but their data do not seem convincing either on the ground of Burk's criticisms (Burk, 1942). The data of Nakatani can probably be explained by the circumstance that this early investigator could not express his determinations on a per cell or unit of DNA basis. In consequence the anaerobic glycolysis of tissue depleted in weight by the diet but unchanged in regard to the number of cells could appear as having increased per unit of weight.

Our experiments did not reveal the nature of this increase of glycolysis in tumours. But it is well known that under appropriate conditions and in the presence of certain fortifying factors the homogenates of the normal tissue may ferment as well or even more vigorously than the homogenates of tumours (Meyerhof, 1949 ; Le Page, 1950). It seems, therefore, that the high fermentation of tumours is the reflection of some change in the regulation of intracellular metabolic processes rather than of the general increase in the level of glycolytic enzymes. The circumstance of increased anaerobic glycolysis coinciding with massive proliferation of the cells suggests that further increase in glycolysis is due only to the

increase in the number of malignant cells. In other words in each of these cells the metabolic change is complete and maximal. This would allow to estimate the number of malignant cells present in a given piece of tissue simply from the rate of anaerobic glycolysis and DNA content.

In conclusion one may say that there can hardly be any doubt that the respiratory damage of the tumor cell is well established. However, inasmuch as the carcinogen does not induce the respiratory damage directly, the mechanism of carcinogenesis needs further elucidation.

SUMMARY

1. Experimental evidence was given to show that the carcinogenic compounds 3′-methyl,4-dimethyl aminoazobenzene and 3,4 benzpyrene are not respiratory inhibitors, although their accumulation inside the cell leads to a marked lowering of respiration, especially when the respiration is expressed per unit of DNA.

2. The respiratory damage is due to the reduction in the amount of mitochondria per cell.

3. Mitochondria and the ergastoplasm-bound RNA are regulated by a common factor which in the liver is influenced by a carcinogen (3′-Me, 4-DAB) in a way opposite to the action of anterior pituitary hormones in their target organs.

4. There is a critical period at which the anaerobic glycolysis suddenly increases, a shift in the distribution of cytoplasmic ribonucleic acid takes place in favour of the unorganized supernatant form and a shift in the protein-free polarographically active sulphydryl groups is observed.

5. This critical period marks the massive proliferation of cells.

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